UNIVERSIDAD AUTÓNOMA DE MADRID

Facultad de Ciencias
Departamento de Química-Física Aplicada

ESTUDIO DEL CONSUMO MODERADO DE VINO SOBRE LA FUNCIÓN DIGESTIVA: METABOLITOS FENÓLICOS Y METABOLOMA FECAL, MICROBIOTA ORAL Y COLÓNICA, Y RESPUESTA INMUNE

IRENE MUÑOZ GONZÁLEZ

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CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN

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Memoria presentada por

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RESUMEN/ABSTRACT

1. RESUMEN

Con el objetivo global de avanzar en el conocimiento de cómo el consumo moderado de vino puede contribuir al mantenimiento de la salud digestiva, en la presente Tesis Doctoral se ha investigado su efecto sobre el metabolismo de polifenoles y la microbiota de la cavidad oral y del colon, así como en otros aspectos relevantes relacionados con la función digestiva humana.

El desarrollo experimental de la Tesis ha englobado un estudio de intervención en humanos y diversos ensayos empleando modelos *in vitro*. El estudio de intervención consistió en el consumo moderado y regular (4 semanas) de vino tinto por un amplio número de sujetos sanos (n=42). Las muestras fecales recogidas se analizaron siguiendo diferentes aproximaciones análiticas (UPLC-ESI-TQ MS/MS y UPLC-ESI-TQ MS/MS), consiguiendo identificar un gran número de metabolitos de origen fenólico y de otras rutas metabólicas. Además, se ha encontrado que el contenido total de metabolitos fenólicos en heces consiguió clasificar a los individuos en función de su capacidad para metabolizar los polifenoles del vino: metabolizadores altos, moderados y bajos. Esta clasificación ha permitido discernir, mediante el análisis metagenómico de las muestras fecales, diferencias en la composición de las poblaciones microbianas intestinales, lo que indica que una diferente composición de la microbiota intestinal podría asociarse con una diferente capacidad para metabolizar los polifenoles del vino. Por otro lado, se ha llevado a cabo el análisis de marcadores inmunes en las mismas muestras, encontrándose una importante reducción en varias citoquinas tras el consumo de vino, en aquellos voluntarios que *a priori* mostraban un elevado contenido fecal en citoquinas.

Empleando un modelo de biopelícula oral de la placa dental supragingival, se ha encontrado que el vino tinto y el vino tinto desalcoholizado inhiben el crecimiento de alguna especies representativas de la microbiota oral como *F. nucleatum* y *S. oralis*. Esta tesis también recoge la primera aplicación a alimentos del sistema SIMGI, un simulador *in vitro* del tracto gastrointestinal de reciente desarrollo. Con ello, se ha logrado un estudio relacional de los metabolitos generados a partir de los polifenoles del vino y otros metabolitos indicativos de la actividad microbiana (ión amonio, SCFAs) con cambios en algunos grupos microbianos. En conjunto, la realización de estudios *in vitro* e *in vivo* de forma conjunta nos ha permitido tener una visión global acerca de los efectos de un consumo moderado de vino sobre la salud digestiva, prestando especial atención en la interacción MICROBIOTA-POLIFENOLES.

1. ABSTRACT

With the overall objective of improving our knowledge of how moderate wine consumption may help to maintain digestive health, in this PhD thesis it has investigated its effect on the metabolism of polyphenols and the microbiota of the oral cavity and colon, as well as other relevant aspects related to the human digestive function.

Experimental development of the thesis has included an intervention study in humans and various assays using *in vitro* models. The intervention study consisted of a regular and moderate consumption (4 weeks) of red wine by a large number of healthy subjects (n = 42). Fecal samples collected were analyzed using different analytical approaches (TQ UPLC-ESI-MS/MS and TQ UPLC-ESI-MS/MS), obtaining the identification of many metabolites of phenolic origin and from other metabolic pathways. Furthermore, it is found that the total content of phenolic metabolites in faeces allowed classification of individuals according to their ability to metabolize wine polyphenols: high, moderate and low metabolizers. This classification has discerned, through metagenomic analysis of fecal samples, differences in the composition of the intestinal microbial populations, indicating that a different composition of the intestinal microbiota could be associated with a different ability to metabolize wine polyphenols. On the other hand, it has been carried out the analysis of immune markers in the same samples, finding a significant reduction in several cytokines after wine consumption, only for those volunteers who had high content fecal cytokines *a priori*.

Using a model of the supragingival oral biofilm of dental plaque, it has been found that red wine and desalcoholized red wine inhibit the growth of some representative species of the oral microbiota as *F. nucleatum* and *S. oralis*. This thesis also includes the first application of the SIMGI system, an *in vitro* gastrointestinal tract simulator recently developed. With this, a study relating metabolites from wine polyphenols and other metabolites indicative of microbial activity (ammonium ion, SCFAs) with changes in some microbial groups has been made. Overall, all together *in vitro* and *in vivo* studies have allowed us to have an overview about the effects of moderate wine consumption on the digestive health, paying particular attention to MICROBIOTA-POLYPHENOLS interaction.

INTRODUCCIÓN

2. INTRODUCCIÓN

2.1. Vino y salud humana

El vino es, según la *Ley 24/2003, de 10 de julio, de la Viña y del Vino*, el alimento natural obtenido exclusivamente por fermentación alcohólica, total o parcial, de uva fresca, estrujada o no, o de mosto de uva. El vino está compuesto principalmente por agua (80-85%), etanol (9-20%), y otros compuestos minoritarios como azúcares, ácidos, compuestos fenólicos, compuestos nitrogenados, compuestos volátiles, lípidos, vitaminas y sales minerales, entre otros, los cuales son los responsables de conferir al vino sus principales características desde el punto de vista organoléptico, nutritivo y saludable.

A pesar de su contenido en etanol, está ampliamente aceptado que el consumo moderado de vino tinto tiene efectos beneficiosos para la salud en el marco de una dieta y estilo de vida saludables, como lo demuestran numerosos estudios científicos cuyo desencadénate principal fue el proyecto MONICA (Multinational MONItoring of trends and determinants in CArdiovascular disease), realizado entre los años 1985 y 1991, que dio lugar a lo que se conoce hoy cómo "paradoja francesa" (Renaud y Lorgeril, 1992). El proyecto MONICA fue un estudio epidemiológico coordinado por la OMS en el que participaron alrededor de 30 centros de investigación europeos y se analizaron los datos procedentes de más de 170.000 individuos acerca de la incidencia de cardiopatías. Una de sus conclusiones principales fue que, a pesar de su dieta rica en grasas saturadas, la población francesa mostró un menor índice de mortalidad por enfermedades cardiovasculares, lo que atribuyó al hábito de beber vino tinto de forma moderada como parte de la dieta.

A partir de ese momento, y hasta hoy, se han llevado a cabo un gran número de estudios con el objetivo de confirmar y fundamentar los efectos beneficios del consumo moderado de vino sobre la salud humana. Así, estudios epidemiológicos posteriores como el de Wollin y col. (2001), han puesto de manifiesto que el consumo moderado de vino reduce de forma considerable el riesgo de padecer enfermedad coronaria. Recientemente, el estudio a nivel nacional PREDIMED (PREvención con DIeta MEDiterránea) sugiere que el vino, consumido y considerado como alimento dentro de la dieta mediterránea, tendría un importante efecto cardioprotector, potenciado por su combinación con una dieta saludable (Estruch y col., 2013). En conjunto, el consumo moderado de vino podría reducir el riesgo de mortalidad por enfermedad coronaria en un 30-40% (Aleixandre y col., 2013), lo que podría atribuirse a una reducción de los distintos factores de riesgo como la ateroesclerosis

(Vinson y col., 2001; Droste y col., 2013), el estrés oxidativo (Micallef y col., 2007; Estruch y col., 2011) o la reducción de mediadores de la inflamación en sangre (Sacanella y col., 2007; Chiva-Blanch y col., 2012).

El consumo moderado de vino también se ha asociado a una menor incidencia de algunas de las enfermedades con mayor prevalencia en la actualidad. A pesar del potencial carcinogénico del etanol, existen evidencias de que el consumo moderado de vino puede disminuir el riesgo de varios tipos cánceres, incluyendo el cáncer de colon, carcinoma de células basales, de ovario, y de próstata (Bianchini y Vainio, 2003). Por ejemplo, Kubo y col. (2009) asociaron un menor riesgo de desarrollar el síndrome de Barrett, precursor del adenocarcinoma de esófago, con el consumo de un vaso de vino tinto al día. Otros autores han demostrado que el consumo moderado de vino podría prevenir la aparición de diabetes de tipo 2, así como sus complicaciones asociadas (Noguer y col., 2012; Chiva-Blanch y col., 2013). Además, el consumo de vino también se ha relacionado con la prevención de enfermedades neurodegenerativas relacionadas con el estrés oxidativo como el Alzheimer o Parkinson (Sun y col., 2008), así como con la reducción de la incidencia de depresión (Gea y col., 2013).

Estos efectos beneficiosos asociados al consumo moderado de vino se han atribuido mayoritariamente a los compuestos fenólicos o polifenoles presentes en esta bebida, cuya descripción se abordará a continuación.

2.1.1. Los compuestos fenólicos del vino

Los compuestos fenólicos son constituyentes naturales de las plantas, encontrándose en los alimentos de origen vegetal. En el caso de la uva, estos compuestos se localizan en las partes sólidas, principalmente en los hollejos, pepitas y raspones. Durante el proceso de vinificación, los compuestos fenólicos pasan al vino, constituyendo uno de los grupos de compuestos mayoritarios en este alimento (Monagas y col., 2005).

Desde el punto de vista químico, el término “polifenol” engloba a un grupo muy heterogéneo de compuestos, que se caracterizan por presentar un anillo aromático al menos con una sustitución hidroxilo y una cadena lateral funcional. Según su estructura química, se subdividen en dos grandes grupos de compuestos: los no flavonoides y los flavonoides. Los compuestos no flavonoideos se caracterizan por presentar un solo anillo de 6 carbonos (C6), siendo los más destacados en este

grupo los ácidos hidroxibenzoicos (C6-C1) e hidroxicinámicos (C6-C3), los alcoholes fenólicos (C6) y los estilbenos (C6-C2-C6). Los compuestos flavonoideos se caracterizan por presentar dos anillos de 6 carbonos unidos por un heterociclo central de 3 carbonos (C6-C3-C6), diferenciándose unos de otros en el grado de oxidación del oxígeno heterocíclico y en la saturación del anillo central. Entre los flavonoides cabe destacar los flavonoles (incluyendo la quercetina, miricetina y el kaemferol así como sus glicósidos) y los flavan-3-oles, principalmente lo monómeros (+)-catequina y (-)-epicatequina, cuyas uniones dan lugar a la proantocianidinas o taninos condensados, localizados en las pepitas y hollejos. Finalmente, se encuentran los antocianos, los cuales son los responsables del color rojo del vino, destacando en este grupo el malvidin-3-glucósido. A modo de ejemplo, la **figura 1** muestra las estructuras químicas de los principales compuestos fenólicos presentes en el vino.

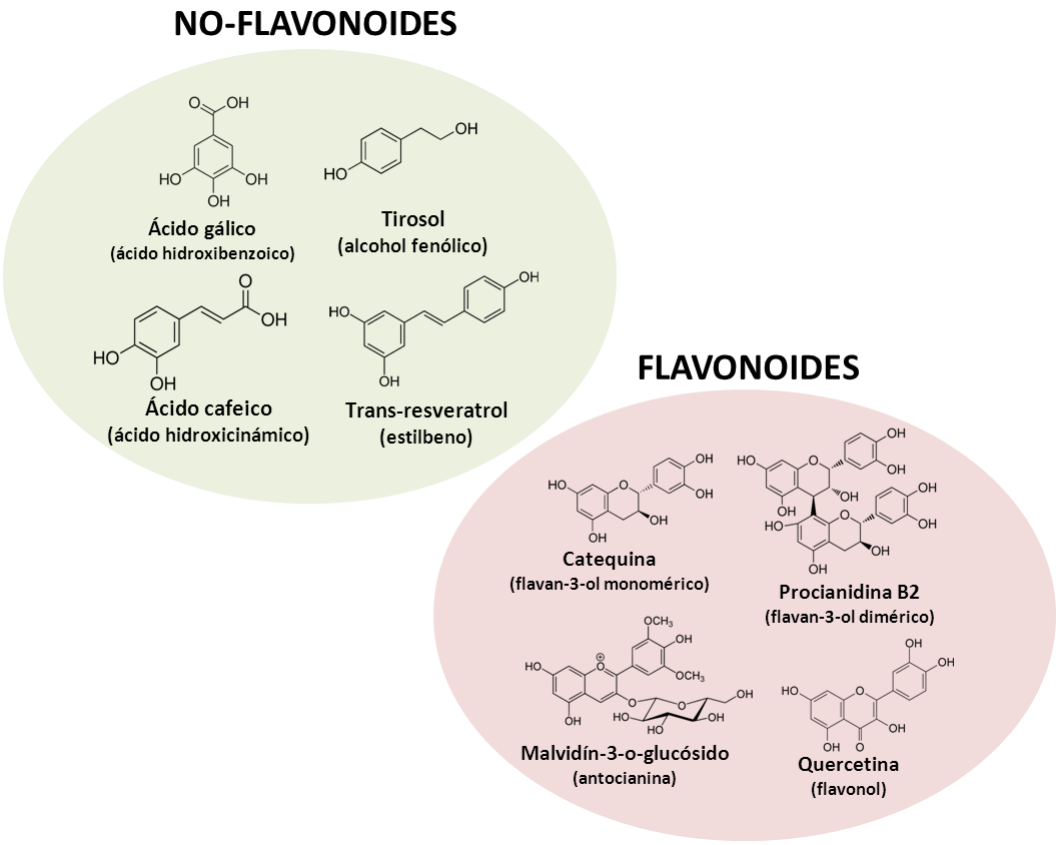

Figura 1. Estructura química de los principales compuestos fenólicos presentes en el vino.

Los polifenoles tienen un gran interés en enología ya que son responsables de muchas de las propiedades organolépticas de los vinos, fundamentalmente el color y la astringencia. El contenido de polifenoles en el vino es variable dependiendo de la variedad de uva, del clima, del tipo de suelo,

y de las prácticas enológicas, entre otros, estimándose en 40-400 mg/L para los vinos blancos y en 900-1400 mg/L para los vinos tintos jóvenes (García-Ruiz y col., 2008).

La **tabla 1** muestra los grupos mayoritarios de compuestos fenólicos que se pueden encontrar en los vinos tintos jóvenes (antocianinas, flavan-3-oles, alcoholes, flavonoles, ácidos y derivados hidroxicinámicos e hidroxibenzoicos y estilbenos) y las concentraciones de los principales compuestos fenólicos identificados dentro de cada grupo.

Tabla 1. Principales compuestos fenólicos identificados en vinos tintos jóvenes (García-Ruiz y col. 2008).

	Concentración (mg/L)		**Concentración** (mg/L)
Ácidos hidroxibenzoicos		**Flavan-3-oles**	
Ácido gálico	10-37	(+)-Catequina	16-58
Ácido protocatéquico	1,2-4,7	(-)-Epicatequina	10-38
Ácido siríngico	4,2-5,8	Procianidinas B1, B2, B3, B4	14-33
Ácidos hidroxicinámicos		**Flavonoles**	
Ácido caftárico	0,7-46	Miricetín-3-glucósidos	1,6-22
Ácido cutárico	0,7-11	Quercetín-3-glucósidos	1,3-34
Ácido caféico	0,3-33	Kanferol-3-glucósidos	Trazas
Ácido *p*-cumárico	0,1-8	Isoramnetin-3-glucósidos	Trazas
		Miricetina	1,7-8
Estilbenos		Quercetina	1,9-15
trans-Resveratrol	0,4-2,5	Kanferol	Trazas
trans-Resveratrol-3-*O*-glucósido	0,1-3	Isoramnetina	Trazas
Alcoholes		**Antocianinas**	
Tirosol	7-26	Delfinidín-3-glucósido	7-11
Triptofol	nd-4,5	Petunidín-3-glucósido	14-25
		Malvidín-3-glucósido	170-260
		Malvidín-3-(6-acetil)-glucósido	23-108
		Malvidín-3-(6-cafeil)-glucósido	3,5-5,6
		Malvidín-3-(6-*p*-cumaril)-glucósido	16-28

nd: no detectado

2.2. Microbiota humana

La microbiota humana es el conjunto de microorganismos presente en los diferentes ecosistemas del cuerpo humano. En su gran mayoría, estos microorganismos no son patógenos y forman parte de la denominada microbiota normal o flora nativa, la cual se encuentra localizada en

cuatro zonas principalmente: piel, boca y tracto respiratorio, tracto urinario y tracto gastrointestinal (**Figura 2**).

De acuerdo con el Proyecto Microbioma Humano, programa de investigación que se inició en 2007 con el objetivo de construir un mapa genético de los microorganismos que forman parte de la microbota humana "sana", se estima que aproximadamente el 90 % de las células del cuerpo humano son de origen bacteriano, vírico o fúngico, no humanas (Turnbaugh y col., 2007). Sin embargo, sólo una fracción de la microbiota humana se ha caracterizado y, mucho menos, identificado. Además se ha estimado que el *microbioma*, conjunto de los genomas de la microbiota humana, podría superar en más de 100 veces al propio genoma humano (Petrosino y col., 2009).

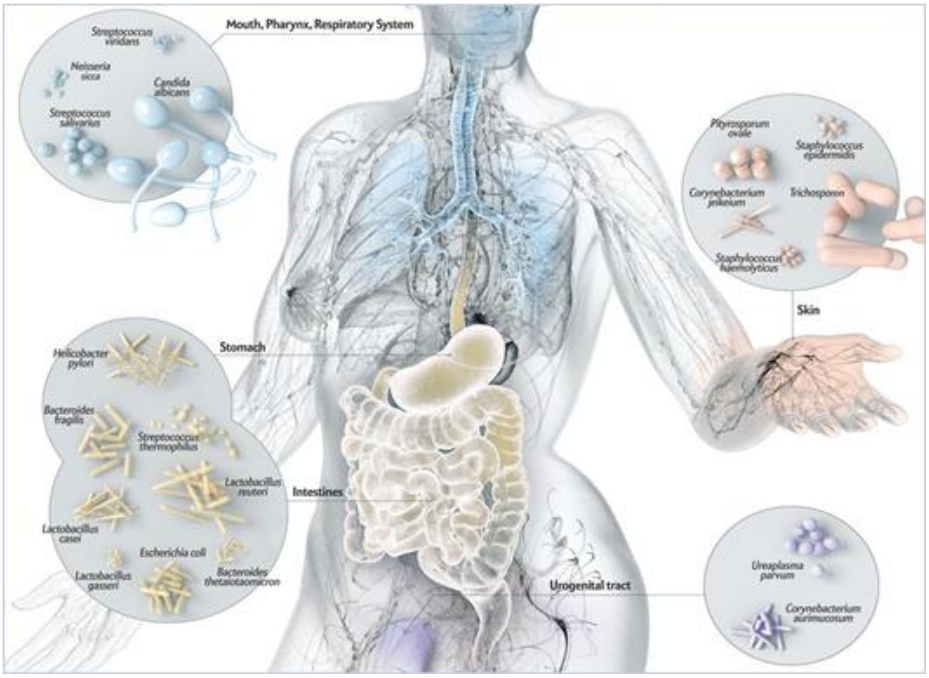

Figura 2. Microbiota humana. Fuente: http://www.scientificamerican.com. Ilustrado por Bryan Christie

La interacción entre la microbiota normal y el ser humano es, en la mayoría de los casos, beneficiosa para ambos, ya que la microbiota participa en numerosos procesos fisiológicos esenciales para mantener la salud humana, como los procesos de digestión y nutrición. La microbiota humana, concretamente la alojada en el tracto gastrointestinal, tiene un papel fundamental en dichos procesos, existiendo una interacción bidireccional MICROBIOTA-NUTRIENTE por la cuál, la microbiota participa en la digestión y metabolismo de los componentes de la dieta y, a su vez, ésta puede verse modulada específicamente por dichos componentes (Maukonen y

Saarela, 2014, Laparra y Sanz, 2010; He y col., 2013). Además la microbiota participa activamente en la defensa y protección frente a patógenos y en el desarrollo de la respuesta inmunológica humana (O'Hara y Shanahan, 2006, Kabat y col, 2014).

En los últimos años, se ha incrementado de forma relevante el número de estudios que buscan estudiar la composición de la microbiota humana y su relación con la salud (**Figura 3**). Ampliar el conocimiento de cómo la microbiota participa el los distintos procesos del cuerpo humano, y del papel de los alimentos a este nivel, podría ser de gran utilidad para paliar o prevenir el desarrollo de ciertas enfermedades, especialmente aquellas relacionadas con la dieta como la obesidad o el síndrome metabolico (DiBaise y col., 2008; Sanz y Moya-Pérez, 2014; Parekh y col., 2014).

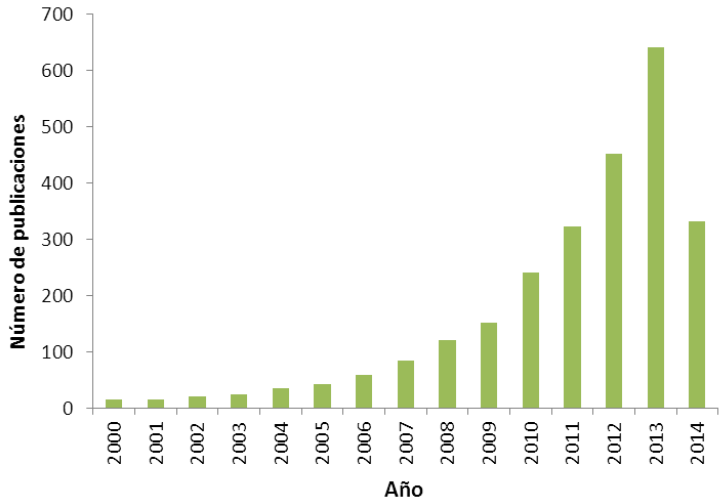

***Figura 3**. Número de publicaciones desde el año 2000 hasta Julio de 2014, registradas en la base de datos Isi Web of Knowledge, utilizando las palabras clave de búsqueda "Human", "Microbiota" y "Health".*

2.2.1. Microbiota de la cavidad oral

La cavidad oral es un complejo ecosistema en el cuál se han detectado más de 750 especies bacterianas distintas, la mayoría no cultivables (Jenkinson y Lamont, 2005). La cantidad y composición de la microbiota oral varía a lo largo de la vida del individuo debido a varios factores como la dieta, el desarrollo de la dentición/pérdida de piezas dentarias, la higiene oral, los hábitos de vida (ej. tabaco, alcohol, etc…). Dentro de la cavidad oral, existen diversos nichos o microambientes con distintas características físicas, químicas y nutricionales, lo que permite el desarrollo de unas u otras especies. Los nichos biológicos son: mucosa, dorso de lengua, superficies dentarias, surco

gingival, materiales bio-compatibles de restauración, ortodoncias, etc., cuya microbiota es distinta, tanto desde el punto de vista cualitativo como cuantitativo (Aas y col., 2005).

La mayor parte de la microbiota oral se organiza y asocia dando lugar a la denominada "biopelícula oral" o "placa dental bacteriana" (**Figura 4**). Esta asociación de las bacterias en forma de biopelícula, permite una interactuación simbiótica, que incluye la co-agregación, (Kolenbrander y col., 2006), el intercambio metabólico, la comunicación entre células (Li y col., 2002), y el intercambio de material genético (Roberts y col., 2010), mecanismos que aumentan la supervivencia de las células y permiten una mayor resistencia frente al estrés mecánico y los tratamientos con antibióticos y otros agentes antimicrobianos (Roberts y col., 2010).

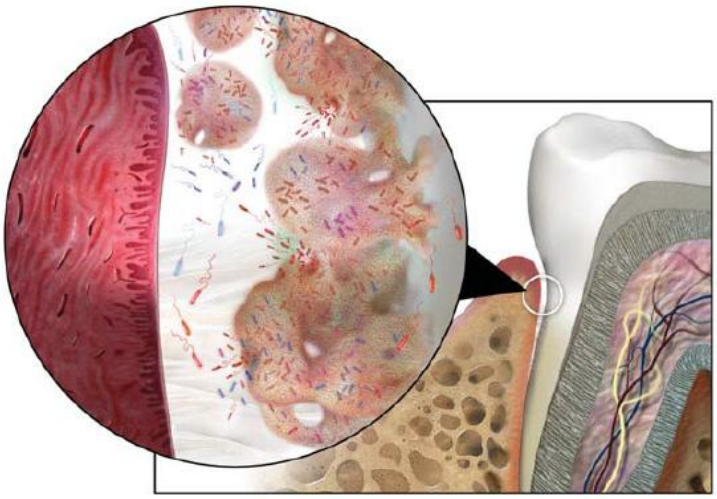

Figura 4. Estructura de la placa dental bacteriana. Fuente: Gurenlian y col. (2007).

En concreto, la placa dental bacteriana consiste en la acumulación heterogénea de bacterias aerobias y anaerobias, rodeadas por una matriz intercelular de polímeros de origen salival y microbiano (dextranos y levanos), que se adhiere al esmalte de los dientes o al espacio gingival dentario (Marsh y Bradshaw, 1995). La placa supragingival es aquella que se ubica en la superficie de los dientes, mientras que la placa subgingival es aquella que se forma en el surco gingival entre diente y encía.

Las bacterias que forman la placa dental son muy variadas pudiéndose encontrar entre 200 y 300 especies diferentes. A modo de ejemplo, la **tabla 2** muestra las principales especies bacterianas que se pueden aislar en la placa dental bacteriana.

Tabla 2. Principales especies que participan en la formación de la placa dental bacteriana (Aas y col., 2005; Marsh, 2009)

GRAM +	
Cocos	**Bacilos**
Streptococcus, Enterococcus, Staphylococcus, Micrococcus, Peptostreptococcus, Peptococcus	*Actinomyces*, Corynebacterium, *Lactobacillus, Bifidobacterium, Gemella, Rothia*
GRAM -	
Cocos	**Bacilos**
Neisseria, Veillonella	*Prevotella, Porphyromonas, Fusobacterium, Tannerella, Treponema, Eubacterium*
OTROS (MINORITARIOS)	
Espiroquetas comensales, hongos y protozoos	

Concretamente, en la placa supragingival, predomina la flora Gram positiva, dominada por anaerobios facultativos. Entre los "colonizadores primarios" de la placa supragingival, destacan los estreptococos como *Streptococcus sanguis, Streptococcus mitis* y *Steptococcus oralis* (Díaz y col., 2006; Dige y col., 2007). Inmediatamente después, se unen otras especies como *Actinomyces*, *Gemella*, *Granulicatella*, *Rothia*, *Neisseria*, *Prevotella* y *Veillonella* (Aas y col., 2005), que también participan en las primeras fases de formación de la biopelícula dental. Tras la multiplicación activa de los "colonizadores primarios", se incorporan otras especies microbianas que dan lugar a la "colonización secundaria" y "colonización terciaria". Durante la formación de la biopelícula, algunas bacterias como *Fusobacterium nucleatum* o determinadas especies de estreptococos juegan un papel esencial, ya que poseen mecanismos para la co-adhesión entre especies (Edwards y col., 2007; Nobbs y col., 2009). La **figura 5** muestra de forma gráfica el proceso de formación de la biopelícula dental.

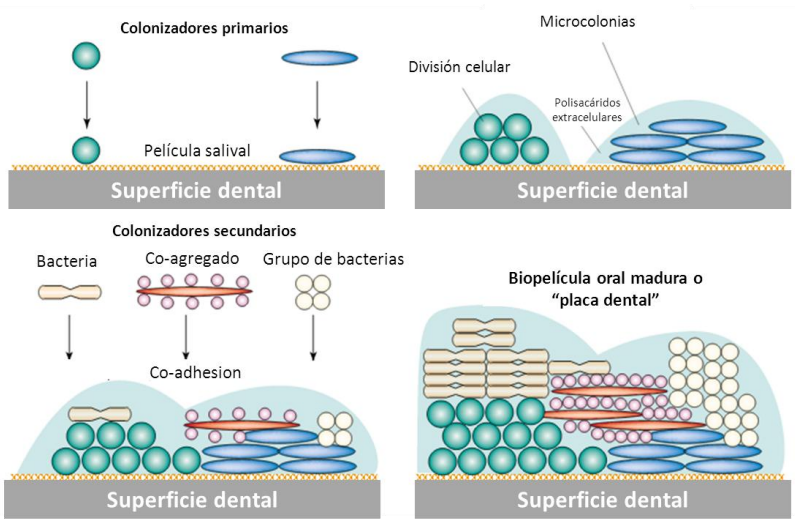

Figura 5. Proceso de formación de la biopelícula dental. Adaptado de Rickard y col. (2003).

2.2.1.1. Importancia de la microbiota oral en la salud humana

La composición de la microbiota de la cavidad oral juega un papel esencial en el mantenimiento de la salud oral y, por extensión, en la salud humana. Es importante destacar que en la cavidad oral, además de las bacterias residentes, se localizan un reservorio de bacterias patógenas que pueden causan infecciones en el tracto respiratorio (Paju y Scannapieco, 2007; Zuanazzi y col, 2010). El equilibrio entre las bacterias que conforman la microbiota normal y las patógenas puede modificarse debido a factores como la edad, la dieta, el estrés, provocando la aparición de ciertas enfermedades. De hecho, está demostrado que la composición bacteriana oral influye directamente en la resolución de algunas de las enfermedades de mayor prevalencia, como son la caries dental y la periodontitis (Kumar, 2013). La desestabilización de la microbiota oral normal, además de otros factores, podría desencadenar el desarrollo de este tipo de enfermedades. La **figura 6** muestra, a modo de resumen, las causas y microorganismos implicados en el desarrollo de la caries dental y la enfermedad periodontal.

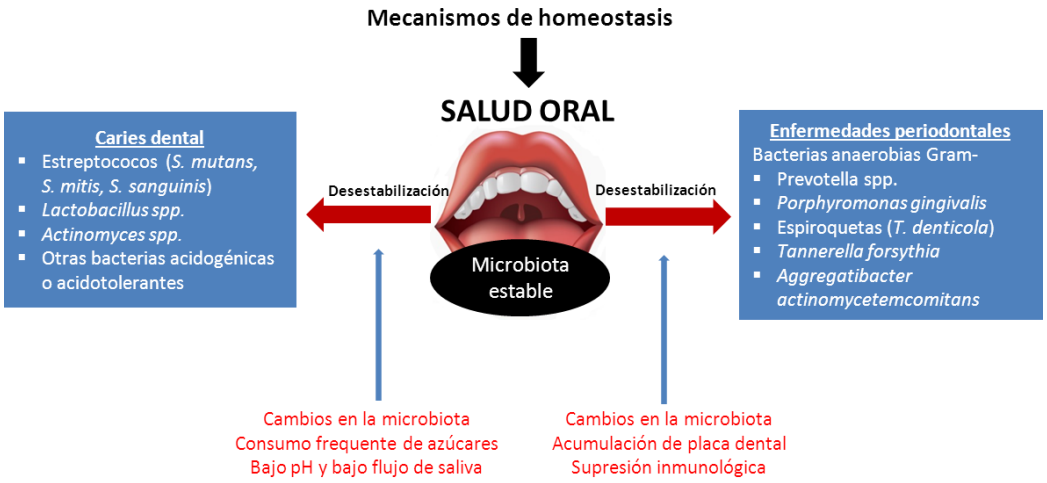

Figura 6. Factores etiológicos de la caries dental y las enfermedades periodontales.

La caries dental podría definirse como la destrucción localizada de los tejidos duros del diente, por la acción bacteriana. Su desarrollo tiene lugar como consecuencia de la fermentación de los carbohidratos ingeridos a través de la dieta por parte de algunas bacterias orales, tales como estreptococos o lactobacilos (Marsh, 2006). Los ácidos formados tras dicha fermentación aceleran la desmineralización de la superficie del diente, contribuyendo a la destrucción del tejido dentario. La enfermedad periodontal es un proceso infeccioso de la encía, producido por diversos microorganismos que colonizan el área supra y subgingival, y caracterizado por la pérdida

estructural del aparato de inserción (o encía) (Armitage, 2000). Según la OMS, la caries dental y la periodontitis son las enfermedades de la cavidad oral más prevalentes en los humanos, y afectan hasta un 60-90 % de la población mundial.

En la actualidad, se aplican diferentes terapias para el control de la placa dental bacteriana y, por tanto, para la prevención y/o tratamiento de las enfermedades asociadas a la cavidad oral. La eliminación mecánica de la placa bacteriana es la técnica más utilizada pero únicamente permite una eliminación parcial de la placa. Otro método ampliamente utilizado son los agentes antimicrobianos, que se suelen aplicar, de forma complementaria a los tratamientos mecánicos, para controlar la formación de la placa dental (Furiga y col., 2008; Marsh, 2010). Hasta la fecha, se han utilizado numerosos agentes antimicrobianos como aceites esenciales procedentes de plantas, el fluoruro de amina, el triclosán, etc. Sin embargo, el agente anti-placa más utilizado y potente es la clorhexidina (Herrera, 2013). A pesar de ser un buen agente anti-placa, la utilización de clorhexidina se ha asociado con algunos efectos secundarios, por ejemplo, la reducción de la percepción del gusto y la pigmentación de los tejidos orales, lo que limita su aplicación en productos de higiene oral. Debido a esto, en los últimos años ha crecido el interés por la búsqueda de nuevos agentes antimicrobianos, preferiblemente productos y/o extractos de origen natural, que carezcan de efectos secundarios y sean activos frente a la placa dental bacteriana, la cual es más resistente a cualquier tratamiento antimicrobiano debido a la presencia de sustancias poliméricas extracelulares, que reducen la penetración de los agentes antimicrobianos, y a la presencia de células persistentes que sobreviven a bajas tasas metabólicas (Hoyle y Costerton, 1991). Por ello, y para comprobar la eficacia de los nuevos agentes antimicrobianos, son necesarios estudios en modelos de biopelícula que incluyan microorganismos normalmente presentes en la cavidad oral y que respeten la estructura asociativa de los mismos. Además, dichos modelos han de ser fácilmente reproducibles y fiables a la hora de comprobar la eficacia de los agentes antimicrobianos.

2.2.1.2. Modelos de simulación de la microbiota oral

Existen varios modelos de simulación de la placa dental bacteriana que pueden ayudar a la hora de estudiar su comportamiento frente a diferentes situaciones y/o tratamientos.

Los modelos estáticos de biopelícula, bien de una sola especie o multi-especies, consisten en la formación de la biopelícula sobre una superficie sólida determinada (hidroxiapatita, vidrio, etc), que permite la adhesión bacteriana, mediante la incorporación de la/las bacterias de interés y su incubación en medios de crecimiento, de forma estática y sin flujo (Guggenheim y col., 2004) (**Figura 7A**). Debido a su sencillez, este tipo de modelos han sido muy utilizados en la evaluación de la eficacia de nuevos tratamientos y/o agentes antimicrobianos para la eliminación y/o control de la placa dental. Por ejemplo, Ruiz-Linares (2014) utilizaron un modelo de biopelícula mono-especie de *S. mutans* para evaluar la eficacia de varios agentes antimicrobianos como chlorhexidina, alexidina y cetrimida. Otros autores han utilizado modelos de biopelícula multi-especie con el fin de testar agentes antimicrobianos como el xilitol (Badet y col., 2008) o distintas formulaciones para pastas dentífricas (Ledder y col., 2012).

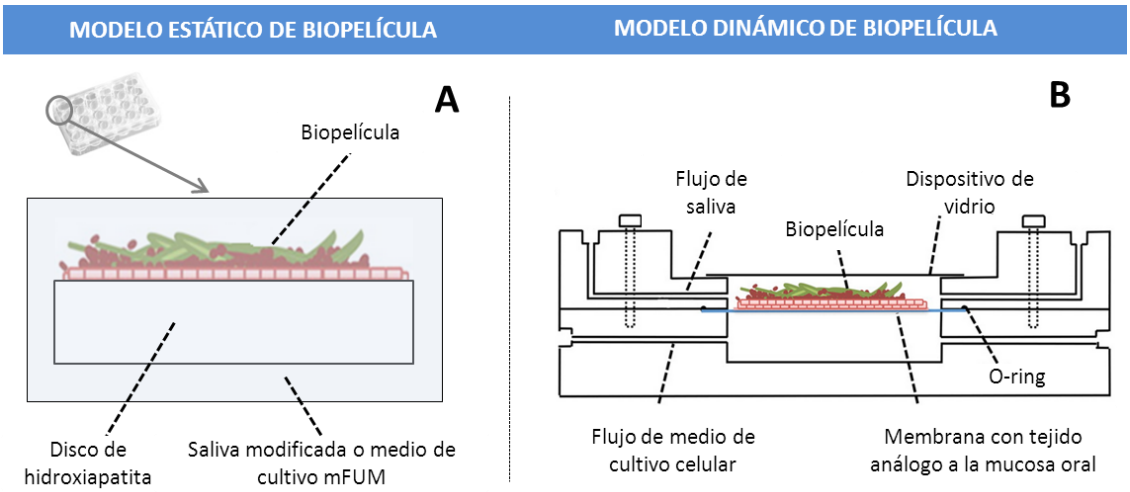

Figura 7. Representación de los modelos estáticos y dinámicos (en cámara de flujo) para la simulación de la biopelícula dental bacteriana. Adaptado de Kolenbrander y col. (2010) y de Diaz y col. (2011), respectivamente.

Otro tipo de modelo son los sistemas dinámicos de cámara de flujo. Estos modelos consisten en una celda de flujo de plástico donde se forma la biopelícula bajo condiciones hidrodinámicas. Estos sistemas permiten una mejor simulación de lo que realmente ocurre en la cavidad oral y resultan especialmente útiles a la hora de realizar análisis estructurales de la formación de las biopelículas (**Figura 7B**). Existen varios estudios que han aplicado este tipo de modelo para comprobar la eficacia de agentes antimicrobianos. Por ejemplo, Blanc y col. (2013) utilizaron un modelo dinámico de

biopelícula formado en un sistema de cámara de flujo, con el objetivo de comprobar la eficacia antimicrobiana de varias formulaciones de higiene oral con chlorhexidina.

Recientemente se han desarrollado nuevos modelos, como los dispositivos intra-orales, que permiten una formación *in vivo* de la biopelícula a partir de la microbiota endógena del propio voluntario y bajo las condiciones fisiológicas reales de la cavidad oral. Estos modelos también se han utilizado con el objetivo de comprobar la eficacia de distintos tratamientos antimicrobianos, sobre la microbiota oral. Por ejemplo, García-Caballero y col. (2013) utilizaron un modelo de biopelícula en discos de vidrio alojados en una férula individualizada del arco inferior de los voluntarios, con el fin de probar la actividad antimicrobiana de un enjuague bucal con clorhexidina. Aunque estos modelos son mucho más realistas a la hora de simular la biopelícula oral, la alta variabilidad interindividual en la composición de las biopelículas formadas podría provocar enormes desviaciones en los resultados obtenidos. Además, su aplicación es mucho más restrictiva y compleja, y requiere voluntarios muy implicados con el estudio así como profesionales altamente cualificados.

2.2.1.3. Interacciones de los compuestos fenólicos del vino con la microbiota oral

La información de la que se dispone actualmente acerca de las posibles interacciones entre los polifenoles y la microbiota de la cavidad oral es escasa. Cabe destacar que la digestión de los alimentos se inicia en la boca con la masticación y es en este paso donde tiene lugar la liberación de los compuestos fenólicos de la matriz alimentaria. Ya en la boca, los compuestos liberados pueden empezar a degradarse debido a la actividad hidrolítica de la saliva. En el caso del vino, el posible metabolismo de sus componentes por parte de la microbiota oral ha sido apenas estudiado. Uno de los pocos estudios que existen en este ámbito es el de Walle y col. (2005), en el que los autores monitorizaron la degradación de glucósidos de flavonoides en sus correspondientes agliconas, por acción de la microbiota oral. Kamonpantana y col. (2012) estudiaron la degradación de antocianinas en presencia de saliva humana y a la temperatura normal de la cavidad oral (37 °C) y demostraron que dicha degradación por parte de la microbiota oral era dependiente de la estructura química, y era parcialmente suprimida después de un tratamiento de la biopelícula con clorhexidina.

Por otro lado, varios autores han demostrado las propiedades antimicrobianas de los compuestos fenólicos o polifenoles frente a bacterias orales aisladas (Jayaprakasha y col., 2003; Ozkan y col., 2004; Cueva y col., 2010). Algunos estudios con polifenoles del té y de arándanos han

demostrado un efecto inhibidor sobre la formación de biopelículas mono-especie de patógenos tales como *S. mutans, S. oralis, S. sobrinus y Porphyromonas gingivalis* (Bodet y col., 2008). Recientemente, se ha demostrado que algunos extractos fenólicos procedentes de vino y de uva, así como extractos fenólicos procedentes de orujo, inhiben el crecimiento de algunas especies asociadas a la formación de la caries dental (Thimothe y col., 2007; Furiga y col., 2009). Sin embargo, hasta el momento no se han llevado a cabo estudios empleen modelos de biopelícula multi-especie para evaluar la capacidad antimicrobiana del vino y de sus polifenoles.

2.2.2. Microbiota intestinal

El intestino es el mayor reservorio de bacterias de todo el cuerpo humano, constituido por cerca de 1000 especies. La concentración de microorganismos en el intestino oscila entre 10^3 y 10^{12} UFC/g de contenido intestinal, alcanzado las concentraciones más elevadas en el colon y las heces, donde puede alcanzar niveles entre 10^{10} y 10^{12} UFC/g de heces (Dethlefsen y col., 2006). Este ecosistema microbiano del intestino incluye tanto especies nativas que colonizan permanentemente el tracto gastrointestinal, como una serie variable de microorganismos que se encuentran de forma transitoria en el tubo digestivo. Las bacterias nativas se adquieren al nacer y durante el primer año de vida, mientras que las bacterias en tránsito se adquieren a lo largo de la vida y de forma continua a través de los alimentos, bebidas u otras fuentes (Guarner, 2007). En la edad adulta, la composición de la microbiota intestinal es relativamente estable a nivel de especie y predominan fundamentalmente los microorganismos anaerobios. Así, los cuatro filos bacterianos dominantes en el intestino adulto son Firmicutes, Bacteroidetes, Actinobacteria y Proteobacteria, siendo los más predominantes los dos primeros (Eckburg y col., 2005; Zoetendal y col., 2006).

A nivel de género, domina el género *Bacteroides,* que constituye aproximadamente un 30% del total de las bacterias en el intestino, seguido por los géneros *Clostridium, Prevotella, Eubacterium, Ruminococcus, Fusobacterium, Peptococus* y *Bifidobacterium*. También están presentes los géneros *Escherichia* y *Lactobacillus*, pero en mucha menor proporción (Beaugerie, 2004). La **figura 8** muestra, a modo de resumen, la composición cualitativa y cuantitativa de la microbiota intestinal.

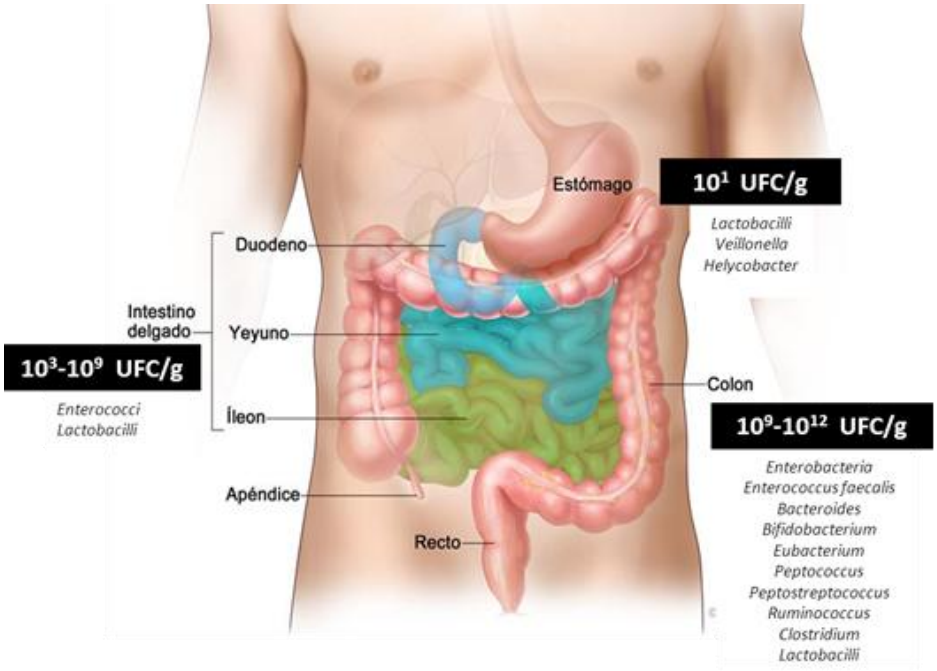

Figura 8. Composición de las poblaciones bacterianas en los distintos segmentos el tracto gastrointestinal.

A pesar de que muchas de las especies de la microbiota intestinal no se han identificado aún, el desarrollo de metodologías de biología molecular y estudios metagenómicos están permitiendo grandes avances en el conocimiento de estas poblaciones tan complejas. Estos estudios están determinando que existe una gran variabilidad interindividual en cuanto a la cantidad y el tipo de bacterias que componen la microbiota intestinal. Así, centrándonos en dicha variabilidad, el consorcio Europeo MetaHit ha establecido la existencia de tres enterotipos de microbiota intestinal en individuos sanos: Enterotipo 1, donde predomina el género *Bacteroides*; Enterotipo 2, en el cual predomina el género *Prevotella* y, finalmente, el Enterotipo 3, predominado por el género *Ruminococcus* (Arumugam y col., 2011).

Actualmente se reconoce que la implantación de las bacterias y su grado de colonización del intestino está ampliamente influenciada por las características de la dieta (Mai, 2004; Scott y col., 2013), así como por factores genéticos y otros inherentes al hábitat, como el pH, la concentración de oxígeno y la biodisponibilidad de los nutrientes (DiBaise y col., 2008). Por ello, es importante conocer qué componentes de la dieta son capaces de modificar la composición de la microbiota humana y cuáles son los mecanismos de acción y las rutas metabólicas implicadas. Son numerosos los estudios que apuntan a los compuestos fenólicos como uno de los grupos de componentes que pueden afectar a la colonización y a la composición de la microbiota intestinal (Dueñas y col., 2014).

Por tanto, mejorar el conocimiento de la microbiota y de la influencia que algunos componentes de la dieta, como los polifenoles, ejercen sobre la misma, permitiría establecer estrategias de alimentación que, mediante la modulación de la microbiota intestinal, contribuyan a la prevención de ciertas enfermedades.

Con el fin de ampliar el conocimiento acerca de la composición de la microbiota intestinal, la mayoría de los estudios que se estan llevando a cabo en la actualidad, están basados en el análisis de las heces, ya que su recolección es sencilla y no invasiva. Diferentes autores como Zoetendal y col. (2002) y Eckburg y col. (2005) han demostrado que la comunidad bacteriana presente en las heces, parece ser similar y representativa de las comunidades bacterianas presentes en el colon. El análisis de las poblaciones bacterianas en muestras fecales, puede realizarse mediante técnicas microbiológicas convencionales basadas en la identificación fenotípica. Sin embargo, estas técnicas son muy restrictivas ya que existen un gran número de especies que no son cultivables. En este caso, la identificación molecular basada en el análisis del gen 16S rRNA mediante técnicas moleculares como la electroforesis en gel por gradiente desnaturalizante (DGGE), la hibridación fluorescente *in situ* (FISH), la PCR cuantitativa (q-PCR) o la secuenciación capilar por el método de Sanger (Zoetendal y col., 2008), son de gran utilidad. Sin embargo, para llevar a cabo un análisis más complejo de la microbiota intestinal, están en auge las técnicas de secuenciación masiva de última generación como la piro-secuenciación, que consiste en un método de secuenciación de ADN basado en la monitorización a tiempo real de la síntesis de ADN. Gracias a este tipo de técnicas, ha surgido el concepto de "metagenómica", definida como el estudio de metagenomas, es decir, del conjunto los genomas de todos los microorganismos en un medio ambiente determinado.

2.2.2.1. Funciones de la microbiota intestinal y su importancia en la salud

La microbiota intestinal juega un papel esencial en el mantenimiento de la homeostasis y en la obtención de energía y nutrientes esenciales tales como ácidos grasos de cadena corta (AGCC), vitaminas (por ejemplo, vitamina K, vitamina B12 y ácido fólico) y aminoácidos, que los seres humanos son incapaces de producir ellos mismos, a partir de la fermentación microbiana de polisacáridos no digeribles de la dieta, tales como almidón resistente o fibra dietética (Tsai y Coyle, 2009) (**Figura 9**). De hecho, algunos autores ha sugerido que la microbiota podría actuar como un

"super órgano" similar al hígado gracias a su amplio abanico de actividades enzimáticas (Bakhed, 2004; O'Hara y Shanahan, 2006).

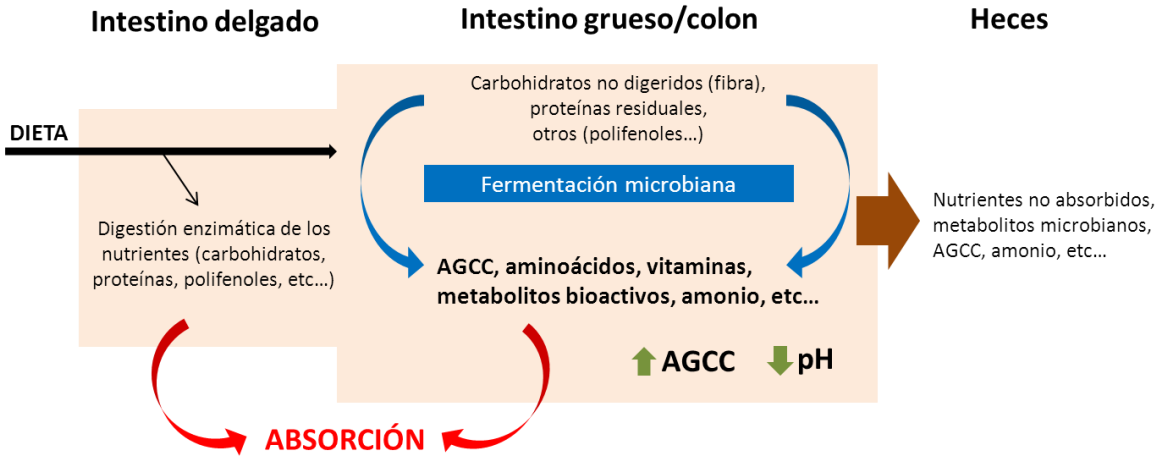

Figura 9. Fermentación microbiana de los componentes no digeribles de la dieta.

La microbiota intestinal tiene además un importante papel en el metabolismo y re-circulación de ácidos biliares, transformación de compuestos con potencial carcinogénico (aminas heterocíclicas), así como la formación de compuestos bioactivos como los metabolitos fenólicos y fitoestrógenos, entre otros (DiBaise y col., 2008). Un estudios llevado a cabo con roedores demostró que en un ambiente estéril, libre de gérmenes, los sujetos no adquirían una microbiota intestinal normal, lo que provocaba, entre otros, un desarrollo corporal anormal con pared intestinal atrófica y motilidad alterada, un metabolismo reducido, corazón, pulmones e hígado de bajo peso, cifras elevadas de colesterol en sangre y un sistema inmune inmaduro; además los sujetos requerían un 30% más de energía en la dieta, y la suplementación de vitamina K y varias vitaminas del grupo B (Tremaroli y col., 2010). Se estima que la liberación de energía por parte de la microbiota comensal es aproximadamente el 10% de la energía absorbida, pero depende en gran medida de la dieta consumida (Savage, 1986). Otra función esencial de la microbiota intestinal es la regulación y el mantenimiento de la estructura intestinal ya que ésta tiene un papel esencial en la proliferación y diferenciación celular, necesaria para mantener la estructura del intestino (Mirón y Cristea, 2012).

Pero quizás, junto con la función de nutrición, la función más importante de la microbiota intestinal es la de protección frente a microorganismos externos patógenos y su participación en el

sistema inmune de la mucosa intestinal. La microbiota intestinal es uno de los elementos más importantes en la barrera de defensa de la mucosa intestinal. Las bacterias comensales compiten con las patógenas por los nutrientes y espacios libres. Por tanto, una microbiota equilibrada es esencial para el mantenimiento de la salud humana y parece proteger frente a trastornos intestinales, enfermedades inflamatorias intestinales, cáncer, etc (Guarner, 2010), por lo que cualquier alteración de la misma podría modificar su carácter protector.

2.2.2.2. Modelos de simulación de la microbiota intestinal

En los últimos años, el desarrollo de modelos de simulación *in vitro* está suponiendo un gran avance en el estudio de las interacciones de la microbiota intestinal y los alimentos y/o compuestos presentes en los mismos (Alminger y col., 2014). La simulación *in vitro* se emplea especialmente para evaluar las transformaciones que sufren los alimentos durante el tracto gastrointestinal. Los modelos que están siendo desarrollados actualmente permiten controlar las distintas variables implicadas en la digestión de los alimentos (tiempos de retención, pH, temperatura, flujo, complejidad y diversidad de la microbiota colónica).

Existen varios modelos de simulación *in vitro* entre los que destacan las fermentaciones estáticas en *batch*, que se llevan a cabo en reactores inoculados con suspensiones de material fecal y mantenidos bajo condiciones anaerobias y a temperatura fisiológica. En los últimos años, estos fermentadores han evolucionado y se han desarrollado simuladores dinámicos del tracto gastrointestinal con el objetivo de simular, en mayor medida, las condiciones reales y los parámetros fisiológicos que pueden influir en la microbiota y su actividad metabólica. Estos simuladores se caracterizan por tener diferentes compartimentos que reproducen las diferentes partes del tracto gastrointestinal (estómago, intestino delgado e intestino grueso). No obstante, debido a su enorme complejidad así como al elevado coste de instalación y puesta a punto, existen muy pocos simuladores de este tipo en el mundo. A nivel europeo, cabe destacar los modelos TIM-1 y TIM-2 (Netherlands Organisation for Applied Scientific Research (TNO), Holanda) (Mateo-Anson y col., 2009; Ribnicky y col., 2014), SHIME (Universidad de Gante, Bélgica) (Kemperman y col., 2013) y, por último, el modelo SIMGI (Instituto de Investigación en Ciencias de la Alimentación (CIAL), Madrid, España) (Barroso y col., 2014), el cual se ha utilizado para llevar a cabo algunos de los estudios de la

presente tesis doctoral. A modo de ejemplo, la **figura 10** muestra un esquema del simulador dinámico del tracto gastrointestinal SHIME.

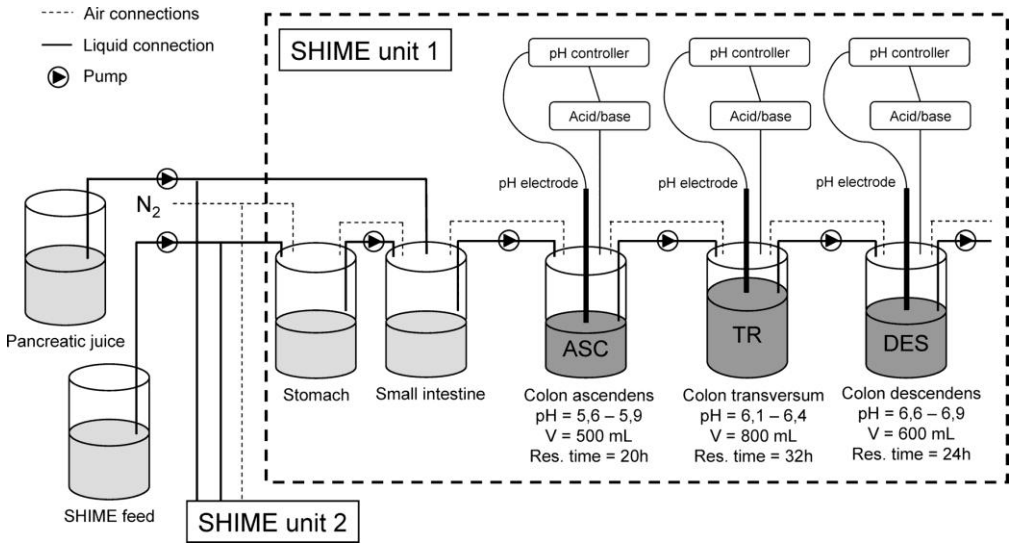

Figura 10. Esquema descriptivo del sistema dinámico del tracto gastrointestinal SHIME.

2.2.2.3. Interacciones de los compuestos fenólicos del vino con la microbiota intestinal

La mayoría de los compuestos fenólicos ingeridos a través de la dieta no son absorbidos en el intestino delgado y se acumulan en el colon (Clifford, 2004). Una vez en el colon, los polifenoles pueden ser metabolizados por la microbiota intestinal, dando lugar a una serie de metabolitos fenólicos bioactivos, los cuales puden producir, a su vez, un efecto de modulación sobre la microbiota colónica (Requena y col., 2010). Esto es lo que se conoce como interacción de doble vía MICROBIOTA-POLIFENOLES (**Figura 11**). Cada vez existen más evidencias del posible efecto de los polifenoles sobre la microbiota intestinal y la repercusión fisiológica *in vivo* de los metabolitos fenólicos resultantes de dicha interacción. Por ello, es necesario realizar estudios que permitan estudiar el efecto del vino sobre las poblaciones bacterianas y a su vez, el metabolismo microbiano de los polifenoles del vino. El análisis de metabolitos en muestras fecales mediante análisis dirigidos y no dirigidos permitía conocer el perfil metabólico global en el lumen intestinal, y por extensión, permitiría avanzar en el conocimiento de cómo la microbiota es capaz de metabolizar los componentes de la dieta.

Figura 11. Interacción bidireccional MICROBIOTA-POLIFENOLES, a nivel intestinal

Aproximadamente el 90-95% de los polifenoles de la dieta llegan al colon, donde son metabolizados por la microbiota intestinal. El catabolismo microbiano de las procianidinas diméricas se inicia con la apertura del anillo central (C), seguida de lactonización, descarboxilación, deshidroxilación, y reacciones de oxidación, entre otros (Monagas y col., 2010). En el caso de flavan-3-oles monoméricos galoilados, el catabolismo microbiano comienza con la rápida escisión del resto éster de ácido gálico por acción de esterasas de origen microbiano, dando lugar a ácido gálico, que se descarboxila y convierte en pirogalol y al monómero correspondiente epicatequina o epigalocatequina. Posteriormente, el anillo central (C) se abre, dando lugar al difenilpropan-2-ol correspondiente, el cual se convierte posteriormente en 5-(3',4'-dihidroxifenil)-γ-valerolactona, en el caso de la epicatequina o en 5-(3',4',5'-trihidroxifenil)-γ-valerolactona en el caso de la epigalocatequina. Posteriormente, el anillo de valerolactona (A) se rompe dando lugar a los ácidos 4-hidroxi-5-(3',4'-dihidroxifenil)valérico o 4-hidroxi-5-(3'-hidroxifenil)valérico, o al ácido 3,4-dihidroxifenilvalérico, el cuál se degrada en otros ácidos fenólicos derivados tales como los ácidos 3,4-dihidroxifenilpropiónico y 3,4-dihidroxibenzoico por la pérdida sucesiva de carbonos de la cadena lateral (**Figura 12**). Con respecto al catabolismo microbiano de flavonoles, éstos pueden transformarse directamente en los ácidos 3,4-dihidroxifenilacético o 3,5-dihidroxifenilacético. En el caso de las antocianinas, éstas se convierten en los ácidos 3,4-dihidroxi, 4-hidroxi-, 3,4-dimetoxi-, y 3-metoxi-4-hidroxi-benzoicos, mediante la sustitución del anillo B de la molécula de antocianina (Aura y col., 2008). Los compuestos no flavonoides como los ésteres hidroxicinámicos (derivados del ácido cafeico) se transforman principalmente en los ácidos 3-hidroxifenilpropiónico, ácido benzoico y 4-etilcatecol.

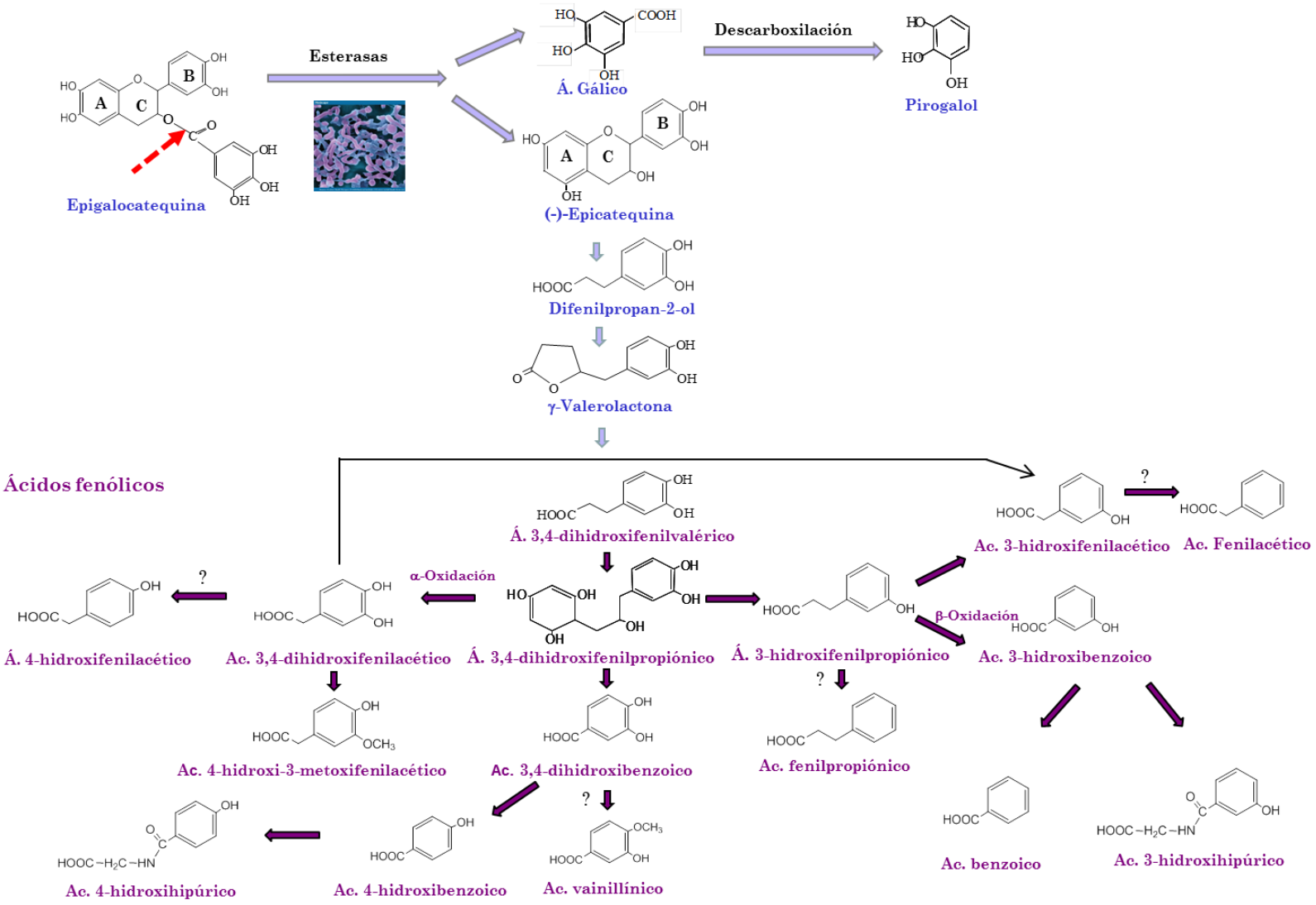

Figura 12. Degradación de flavan-3-oles monoméricos por la microbiota intestinal. Adaptado de Monagas y col. (2010).

Una vez absorbidos, los metabolitos microbianos formados son metabolizados por enzimas de fase II en el hígado dando lugar a metabolitos conjugados (glucurónidos y sulfatos), antes de ser excretados por la orina. Los metabolitos fenólicos no absorbidos son eliminados por las heces por lo que, en consecuencia, el consumo moderado de vino tinto podría promover un aumento significativo de los metabolitos fenólicos en los fluidos biológico (heces y orina). Por tanto, la realización de estudios tanto dirigidos como no dirigidos para analizar el perfil de metabolitos fenólicos en los fluidos biológicos humanos, principalmente en heces, podría permitir un gran avance en el conocimiento de la implicación de la microbiota intestinal en el metabolismo de los compuestos fenólicos del vino. Además, como varios autores han sugerido, estos metabolitos formados durante el tracto gastrointestinal podrían tener una mayor actividad beneficiosa que sus precursores presentes en el vino (Monagas y col., 2010; Williamson y Clifford, 2010).

A pesar de los avances realizados recientemente en el conocimiento de la identificación de los metabolitos fenólicos, las especies de bacterias específicas capaces de metabolizar la mayoría de los polifenoles del vino en el tracto gastrointestinal y las vías de degradación siguen siendo en gran parte desconocidas (Selma y col, 2009, Tabasco y col., 2010)

El avance en el conocimiento de la composición de las poblaciones bacterianas intestinales así como el estudio de las rutas implicadas en la absorción, transformación y metabolización de los polifenoles es esencial para evaluar esta interacción bidireccional. En este ámbito, se han llevado a cabo estudios a distintos niveles (*in vitro*, *in vivo* en animales y estudios de intervención en humanos) prestando especial atención en el efecto de modulación de los polifenoles del vino sobre las diferentes poblaciones bacterianas intestinales (**tablas 3 y 4**). Por ejemplo, varios autores han utilizado modelos de fermentación en *batch*, para evaluar, en conjunto, la estabilidad de los polifenoles en presencia de la microbiota intestinal humana y los cambios en las poblaciones microbianas tras la intervención con compuestos fenólicos puros (Tzounis y col., 2008; Hidalgo y col., 2012), y con extractos ricos en polifenoles, derivados de la uva (Cueva y col., 2012) y del vino (Sánchez-Patán y col., 2012 y Barroso y col., 2013). En general, estos estudios han demostrado el aumento de algunas de las poblaciones bacterianas más relevantes del intestino como *Lactobacillus*, *Enterococcus* y *Bifidobacterium,* y la reducción de otras, principalmente *C. hystoliticum*.

Por otro lado, en un estudio llevado a cabo con un el sistema dinámico de simulación del tracto gastrointestinal SHIME, cuyo objetivo fue monitorizar los cambios en las poblaciones microbianas intestinales tras el tratamiento con un extracto de vino tinto (Kemperman y col., 2013), los autores encontraron cambios destacables en la composición de la microbiota intestinal tras el tratamiento con el extracto de vino.

En cuanto a los estudios *in vivo*, la mayor parte se ha realizado con animales y administrando extractos de vino (Dolara y col., 2005) o de pepita de uva (Viveros y col., 2011). También se han llevado a cabo estudios de intervención en humanos que han utilizado para la intervención un extracto de pepita de uva rico en procianidinas (Yamakoshi y col., 2001) o vino tinto (Queipo-Ortuño, 2012). En este último estudio, que implicó 8 voluntarios, los cuales consumieron vino tinto, vino tinto desacoholizado y ginebra, durante 20 días cada uno, los autores encontraron un aumento en las poblaciones de *Enterococus spp.*, *Prevotella spp.*, *Bacteroides Bifidobacterium spp.*, *Bacteroides uniformis*, *Eggerthella lenta*, y en el grupo *Blautia coccoides-Eubacterium rectale*, y un disminución en *Clostridium spp.* y *C. hytoliticum* group.

Tabla 3. Estudios de *in vitro* para evaluar el efecto de modulación de la microbiota por polifenoles del vino.

Estudios de fermentación en batch								
Referencia	**Concentración fecal**	**Compuesto fenólico o alimento**	**Dosis**	**Tiempo de incubación**	**Técnica de análisis**	**Favorece crecimiento**	**Inhibe crecimiento**	**Sin efecto**
Tzounis y col. (2008)	10 %, w/v	(+)-Catechin	150 mg/L, 1000 mg/L	< 48 h	FISH	*Lactobacillus-Enterococcus* spp. *Bifidobacterium* spp. *Grupo C. coccoides-E. rectale* grupo *E. coli*	*Grupo C. histolyticum*	
Hidalgo y col. (2012)	10 %, w/v	Malvidin-3-*O*-glucosido Mezcla de antocianinas	20 mg/L y 200 mg/L 4850 mg/L y 48500 mg/L	< 24 h	FISH	*Lactobacillu-Enterococcus* spp. *Bifidobacterium* spp. *Grupo C. coccoides-E. rectale*		
Cueva y col. (2012)	10 %, w/v	Fracciones de extracto de pepita se uva	300-450 mg/L	< 48 h	FISH	*Lactobacillus-Enterococcus* spp.	*Grupo C. histolyticum*	
Sánchez-Patán y col. (2012)	1 % w/v	Extracto de vino	600 mg/L	48h	FISH		*Grupo C. histolyticum*	*Lactobacilluss-Enterococcus spp.*
Barroso y col. (2013)		Extracto de vino	500 mg/L	48h	qPCR	*Lactobacillus* spp. *Bifidobacterium* spp. *Bacteroides* spp. *Ruminococcus* spp.		

Estudios con modelos dinámicos de simulación de tracto gastrintestinal								
Referencia	**Simulador**	**Compuesto fenólico o alimento**	**Dosis**	**Tiempo de incubación**	**Técnica de análisis**	**Aumento en la población**	**Reducción en la población**	**Sin efecto**
Kemperman y col. (2013)	Twin-SHIME	Extracto de vino	3 dosis diarias (1000 mg de polifenoles diarios)	2 semanas	Recuento qPCR PCR-DGGE Piroseq.	*Klebsiella* spp. *Alistipes* spp. *Cloacibacillus* spp. *Victivallis* spp. *Akkermansia* spp	*Bifidobacteria* *Blautia coccoides grupo* *Anaeroglobus spp.* *Subdoligranulum spp.* *Bacteroides*	

Tabla 4. Estudios de *in vivo* para evaluar el efecto de modulación de la microbiota por polifenoles del vino.

Estudios *in vivo* con animales								
Referencia	**Animal**	**Compuesto fenólico o alimento**	**Dosis**	**Tiempo de incubación**	**Técnica de análisis**	**Aumento en la población**	**Reducción en la población**	**Sin efecto**
Dolara y col. (2005)	Ratas	Extracto de polifenoles del vino	50 mg/kg	16 semanas	Recuento en placa	Lactobacilli Bifidobacteria	Propionibacteria *Bacteroides* Clostridia	
Viveros y col. (2011)	Pollos	Extracto de pepita de uva	7,2 g/kg dieta (GSE) (acceso libre)	21 dias	Recuento en placa T-RFLP	*E. coli* *Enterococcus* spp. *Lactobacillus* spp.		
Estudios de intervención en humanos								
Referencia	**Número de voluntarios**	**Compuesto fenólico o alimento**	**Dosis**	**Tiempo de incubación**	**Técnica de análisis**	**Aumento en la población**	**Reducción en la población**	**Sin efecto**
Yamakoshi y col. (2001)	9	Extracto de pepita de uva rico en proantocianidinas	0,5 g/día	6 semanas	Recuento en placa	*Bifidobacterium* spp.	Enterobacteriaceae	
Queipo-Ortuño y col. (2012)	10	Vino tinto	272 mL/día	20 dias	qPCR	*Enterococcus* spp. *Prevotella* spp. *Bacteroides* *Bifidobacterium* spp. *Bacteroides uniformis* *Eggerthella lenta* *Grupo C. coccoides-E. rectale*	*Clostridium* spp. *Grupo C. histolyticum*	Actinobacteria

2.3. Respuesta inmune intestinal

El sistema inmunitario humano en el encargado de proteger al organismo frente a la continua amenaza de antígenos de diferente naturaleza. Éste se compone de mecanismos innatos y adaptativos que protegen al individuo frente a antígenos y patógenos externos. Los mecanismos innatos funcionan independientemente de exposiciones previas a agentes infecciosos e incluyen barreras mecánicas (piel, mucosas) y componentes celulares (macrófagos y neutrófilos). Sin embargo, en el sistema inmune adaptativo, los elementos celulares (fundamentalmente linfocitos B y T) y moleculares requieren del contacto previo con el agente desencadenante. Ambos mecanismos, actúan de manera complementaria y conducen a la instauración de la "memoria inmunológica", propiedad a través de la cual, después del contacto inicial con un antígeno, el organismo adquiere la capacidad de responder de una forma más óptima y rápida ante una nueva exposición al mismo antígeno (Wolowczuk y col., 2008).

El sistema inmune de las mucosas, especialmente el asociado con el tracto gastrointestinal, es el más complejo y numeroso del sistema inmune. La mucosa intestinal, concretamente, está formada por el epitelio (enterocitos), que cubre las vellosidades, la lámina propia y el muscularis (**Figura 13**). La mucosa intestinal constituye un sitio de alta actividad inmunitaria ya que recibe diariamente una enorme carga antigénica y es capaz de establecer mecanismos para mantener una mucosa intestinal íntegra y una población bacteriana normal y estable.

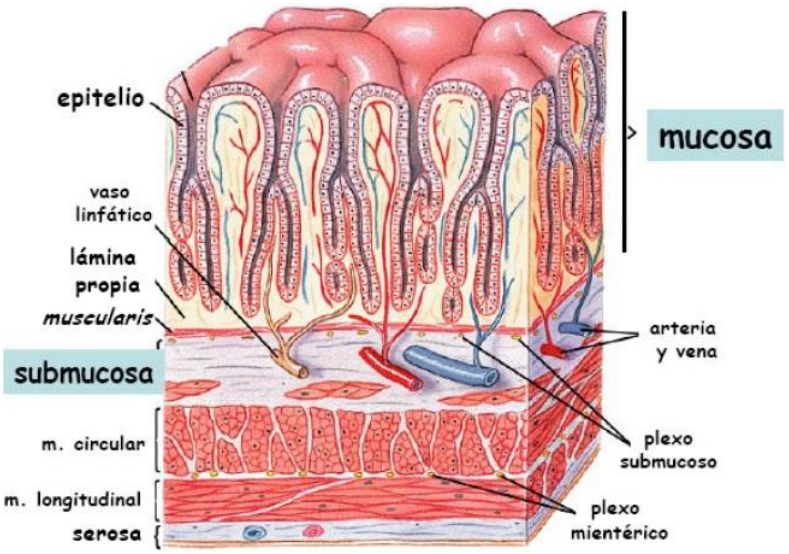

Figura 13. Representación gráfica de la mucosa y submucosa intestinal.

Son numerosos los mecanismos de protección en el tracto gastrointestinal que permiten la protección frente a antígenos y patógenos, destacando algunos como la saliva, el ácido gástrico, las fuertes uniones entre las células epiteliales que sellan los espacios celulares, los movimientos peristálticos, el flujo permanente de moco, los enzimas proteolíticos (lisozima), la flora intestinal, y el sistema inmune intestinal (Isolauri y col., 2001). Sin embargo, la barrera más efectiva está constituida por el tejido linfoide asociado al intestino o GALT (*Gut-Associated Lymphoid Tissue*), caracterizado por su particular estructura diferenciada en tejido *organizado*, inductor de la respuesta inmunitaria intestinal y tejido *difuso*, efector de la respuesta inmunitaria. Los componentes del tejido linfoide *organizado* del sistema inmune (principalmente las placas de peyer) son permeables a la entrada de antígenos y son los responsables de la regulación de la respuesta inmune frente a éstos. A modo de resumen, los antígenos son transportados a su través hasta contactar con las células inmunes (macrófagos/monocitos y linfocitos T y B), que liberan mediadores solubles de la inmunidad entre los que destacan los anticuerpos (inmunoglobulinas) y las citoquinas.

Las citoquinas son un grupo de proteínas de bajo peso molecular que actúan mediando interacciones complejas entre las diferentes células del sistema inmunitario. Éstas tienen un papel esencial en la homeostasis del sistema inmune local y en la regulación de las funciones de barrera y absorción de la mucosa, ya que funcionan como mensajeros del sistema inmune: regulan la intensidad y duración de la respuesta inmune, estimulando o inhibiendo la proliferación de varias células, la secreción de anticuerpos o de otras citoquinas. Las citoquinas pueden ser clasificadas de varias maneras. Por ejemplo, la **tabla 5** muestra su clasificación desde un punto de vista químico/funcional.

Tabla 5. Clasificación de las citoquinas

Grupo	Ejemplos
Interleuquinas	IL 1 to IL 20
Interferones	IFN-α, IFN-ß e IFN-γ
Factores de estimulación de colonias	GM-CSF, G-CSF, M-CSF
Factores de necrosis tumoral	TNF-α, TNF-ß
Factores quinotácticos	MIP, MCP

Sin embargo, hay otras clasificaciones posibles que tienen especial interés a la hora de intentar relacionar el contenido intestinal de citoquinas con la respuesta inflamatoria intestinal. Por ejemplo, las citoquinas han sido clasificadas por varios autores como citoquinas pro-inflamatorias y citoquinas anti-inflamatorias, en función de su participación en la respuesta inflamatoria.

Algunas citoquinas pro-inflamatorias como el TNF-α desempeñan un papel clave en la iniciación de la cascada de otras citoquinas pro-inflamatorias, incluyendo IL-1ß, IL-6, y IFN-γ, que son biomarcadores importantes en los procesos de inflamación (Colombo, 2013). De hecho, según algunos autores, estas citoquinas pro-inflamatorias son los mediadores primarios de los procesos de inflamación en algunas de las enfermedades intestinales caracterizadas por un deterioro de la función de la barrera intestinal (Carol y col., 1998). Varios autores sugieren que el desarrollo de muchas de las enfermedades inflamatorias intestinales (Wall y col., 1999; Ardizzone y col., 2005) podrían estar relacionadas con un desequilibrio en el contenido de citoquinas pro y anti inflamatorias.

La cuantificación de los mediadores de la inflamación, especialmente de las citoquinas pro-inflamatorias en las heces, podría considerarse como un buen método para conocer el estado inflamatorio intestinal de los sujetos y el efecto de una posible intervención nutricional sobre dicho estado. Otros marcadores como la proteína C-reactiva (Vermeire, 2004) y la calprotectina fecal (Lewis, 2011; Ricanek, 2011) han sido ampliamente utilizados como marcadores no invasivos de la inflamación intestinal.

La microbiota intestinal tiene un papel esencial a la hora de defender al organismo frente a patógenos externos. Esta actúa como parte fundamental en las funciones de defensa del organismo, mediante la competencia con las bacterias patógenas así como la secreción de sustancias que inhiben el crecimiento de éstas. Además, existe un permanente estado de comunicación e intercambio de señales entre el huésped y la microbiota, lo que permite por un lado, la regulación del equilibrio entre las diferentes especies de microorganismos que conviven con él y por otra, la respuesta del huésped hacia agentes externos (Werner, 2007). Las interacciones entre microorganismos, epitelio y tejidos linfoides intestinales son múltiples y continuas, de modo que son capaces de modular de manera continua los mecanismos locales y sistémicos de la inmunidad, adaptándolos al ambiente microbiano (Magalhaes, 2007). El epitelio de la mucosa intestinal posee potentes mecanismos de defensa que le permiten

mantener su integridad y la de todo el organismo, al mismo tiempo que confieren capacidad para discriminar entre patógenos y comensales (Salzman, 2007).

De hecho, la inflamación intestinal va normalmente acompañada de un desequilibrio de la microbiota intestinal (Salminen y col., 1998), y en esos casos, son numerosos los microorganismos patógenos que pueden implantarse y provocar el desarrollo de la enfermedad.

El efecto antinflamatorio derivado del consumo moderado de vino se ha evidenciado principalmente en relación con las enfermedades cardiovasculares (Avellone, 2006; Li, 2012; Chiva-Blanch, 2013). Los polifenoles del vino podrían contribuir a la modulación de la respuesta inflamatoria, tal y como se ha demostrado en varios estudios *in vitro* llevados a cabo con compuestos fenólicos puros y extractos fenólicos procedentes del vino (Bognar y col., 2013; Biasi y col., 2013; Nunes y col., 2013). La capacidad anti-inflamatoria de los compuestos fenólicos podría ser tal, que algunos autores han sugerido su utilización como alternativa para prevenir o tratar enfermedades inflamatorias intestinales (García-Lafuente y col., 2009). Sin embargo, y a pesar de su potencial efecto antinflamatorio de los compuestos fenólicos, los mecanismos moleculares responsables siguen siendo, en gran parte, desconocidos. Hasta el momento, los estudios que estudian los efectos de los polifenoles del vino en relación a la inflamación intestinal, se han realizado principalmente con modelos de cultivos celulares (Bognar y col., 2013; Biasi y col., 2013; Nunes y col., 2013). Por lo tanto, son necesarios estudios *in vivo* para avanzar en el conocimiento de los mecanismos de acción de los polifenoles del vino durante la inflamación intestinal.

INTERÉS, HIPÓTESIS Y OBJETIVOS

3. INTERÉS, HIPÓTESIS Y OBJETIVOS

Las evidencias científicas actuales sugieren que el consumo moderado de vino, dentro del contexto de "dieta saludable", contribuye a reducir el riesgo de enfermedades cardiovasculares, cáncer y desordenes neurodegenerativos, así como el envejecimiento prematuro. Muchas de estas propiedades que se atribuyen al vino, se asocian fundamentalmente con el contenido y el tipo de polifenoles, componentes bioactivos mayoritarios en los vinos, especialmente en los vinos tintos.

El estudio de la interacción entre los polifenoles y la microbiota humana de la cavidad oral y del intestino ha cobrado gran interés en los últimos años. El tránsito del vino por la cavidad oral constituye una primera barrera para la biodisponibilidad de sus componentes en el organismo, sin embargo el metabolismo oral de los compuestos fenólicos del vino se desconoce en gran medida. Por otro lado, algunos polifenoles parecen tener un efecto sobre el crecimiento de bacterias orales aisladas, aunque no existen apenas estudios que evalúen sus efectos sobre las principales especies que, en forma de biopelícula, se adhieren a la superficie de los dientes y forman la placa dental. La microbiota intestinal, gracias a sus funciones de adquisición de nutrientes y energía, y de mantenimiento de la homeostasis, marcadas en su genoma (microbioma), contribuye de forma significativa en el metabolismo global del organismo, considerándose que actúa como un "órgano metabólico. Los polifenoles presentes en los alimentos son metabolizados extensivamente durante su paso por el tracto intestinal, de tal forma que muchos de los efectos sobre nuestra salud asociados a su ingesta, se atribuyen a los metabolitos fenólicos producidos por la microbiota intestinal. Descifrar las bases científicas de la relación entre el alimento y la microbiota humana, resulta clave en el estado nutricional y de salud, y en la incidencia de patologías digestivas, metabólicas e inmunológicas.

Gran parte de la investigación sobre la relación entre los alimentos y/o sus componentes, y la microbiota humana está sustentada por estudios en sistemas *in vitro* o en animales modelo, previa a la experimentación en seres humanos. Sin embargo en lo que se refiere a la interacción entre el vino y/o sus componentes (i.e. polifenoles) y la microbiota humana, los estudios de intervención en humanos son muy escasos. Debido a la gran variabilidad inter-individual y a la complejidad de las funciones de la microbiota y su interacción con el huésped, son necesarios estudios de intervención que tengan en cuenta la variabilidad de la microbiota humana entre individuos, así como la ingestión continuada de polifenoles y las implicaciones en la salud intestinal de forma integrada. Por su parte, los sistemas modelo de simulación permiten un mejor control de la experimentación,

considerando las distintas variables que intervienen a nivel fisiológico, como una herramienta de gran valor para los estudios sobre los mecanismos de acción asociados; por ello, en combinación con los estudios de intervención en humanos, constituyen la principal apuesta para establecer bases científicas sólidas de cómo los polifenoles de la dieta, y especialmente del vino, afectan a la función digestiva, a través de su metabolismo e impacto en la microbiota oral e intestinal.

En base a lo expuesto, nuestra **hipótesis** de trabajo es la siguiente:

El consumo moderado de vino podría influir en la composición de las poblaciones microbianas a lo largo del tracto gastrointestinal, principalmente en la boca y el intestino, contribuyendo, por tanto, al mantenimiento de la función digestiva e influyendo positivamente en algunas de las funciones del tracto digestivo como es la respuesta inmune. A su vez, el metabolismo de los polifenoles del vino por parte de dichas poblaciones bacterianas, podría producir metabolitos con efectos beneficiosos a nivel fisiológico, así como modificar el contenido y perfil fenólico en el lumen intestinal, visible también en el metaboloma fecal.

A partir de esta hipótesis, y con el **objetivo general** de profundizar en los efectos del consumo moderado de vino sobre la salud humana, la presente Tesis Doctoral se centra en la función oral e intestinal. Mediante una perspectiva amplia que engloba la ejecución de un estudio de intervención nutricional en humanos y el empleo de nuevos modelos de simulación *in vitro*, pretende evaluar el efecto del consumo moderado de vino en la composición y función de la microbiota oral e intestinal, y su repercusión/impacto sobre el metabolismo fenólico y el metaboloma fecal, y la modulación de la respuesta inmune intestinal.

Los **objetivos concretos** que se han perseguido son:

1. Evaluar los cambios en el metaboloma fecal, el perfil fecal de marcadores inmunológicos y la composición de la microbiota intestinal, tras el consumo moderado de vino en humanos.
2. Elucidar los efectos del vino y sus componentes sobre bacterias de la placa dental, en un modelo de biopelícula de la placa supra-gingival humana.
3. Estudiar los cambios en la población de bacterias intestinales y el metabolismo de polifenoles del vino tinto de estudio en un modelo de simulación *in vitro* del tracto gastrointestinal.

Para la consecución de los objetivos, se ha llevado a cabo el siguiente **plan de trabajo** que también se esquematiza en la **figura 14**:

1. Diseño y ejecución de un estudio de intervención de consumo moderado de vino tinto por un grupo significativamente amplio de voluntarios sanos.
2. Optimización de metodologías para la preparación de muestras fecales (extracción en fase sólida, SPE) y el análisis de metabolitos por técnicas instrumentales avanzadas: UPLC-ESI-TQ MS/MS (análisis dirigido de compuestos fenólicos) y UHPLC-TOF MS (análisis no dirigido de metabolitos).
3. Determinación de metabolitos fenólicos por UPLC-ESI-TQ MS/MS en las muestras fecales humanas recogidas antes y después del consumo de vino tinto, y análisis estadístico de posible estratificación poblacional.
4. Determinación de metabolitos mediante una aproximación metabolómica (UHPLC-TOF MS) en las muestras fecales antes y después del consumo de vino tinto, e identificación de potenciales biomarcadores fecales del consumo de vino.
5. Seguimiento de un amplio número de marcadores inmunológicos en las muestras fecales recogidas antes y después del consumo de vino tinto.
6. Análisis de la composición de la microbiota intestinal humana mediante una aproximación metagenómica, en las muestras fecales recogidas antes y después del consumo de vino tinto.
7. Aplicación del vino de estudio y extractos enológicos relacionados en un modelo de biopelícula de la placa supra-gingival humana oral.
8. Aplicación del vino de estudio en un modelo de simulación *in vitro* del tracto gastrointestinal (SIMGI).

El presente trabajo de investigación ha sido realizado gracias a la financiación del Ministerio de Ciencia e Innovación y el Ministerio de Economía y Competitividad a través de los Proyectos de Investigación AGL2009-13361-C02-01 y AGL2012-04172-C02-01 y a la concesión de una Beca FPI.

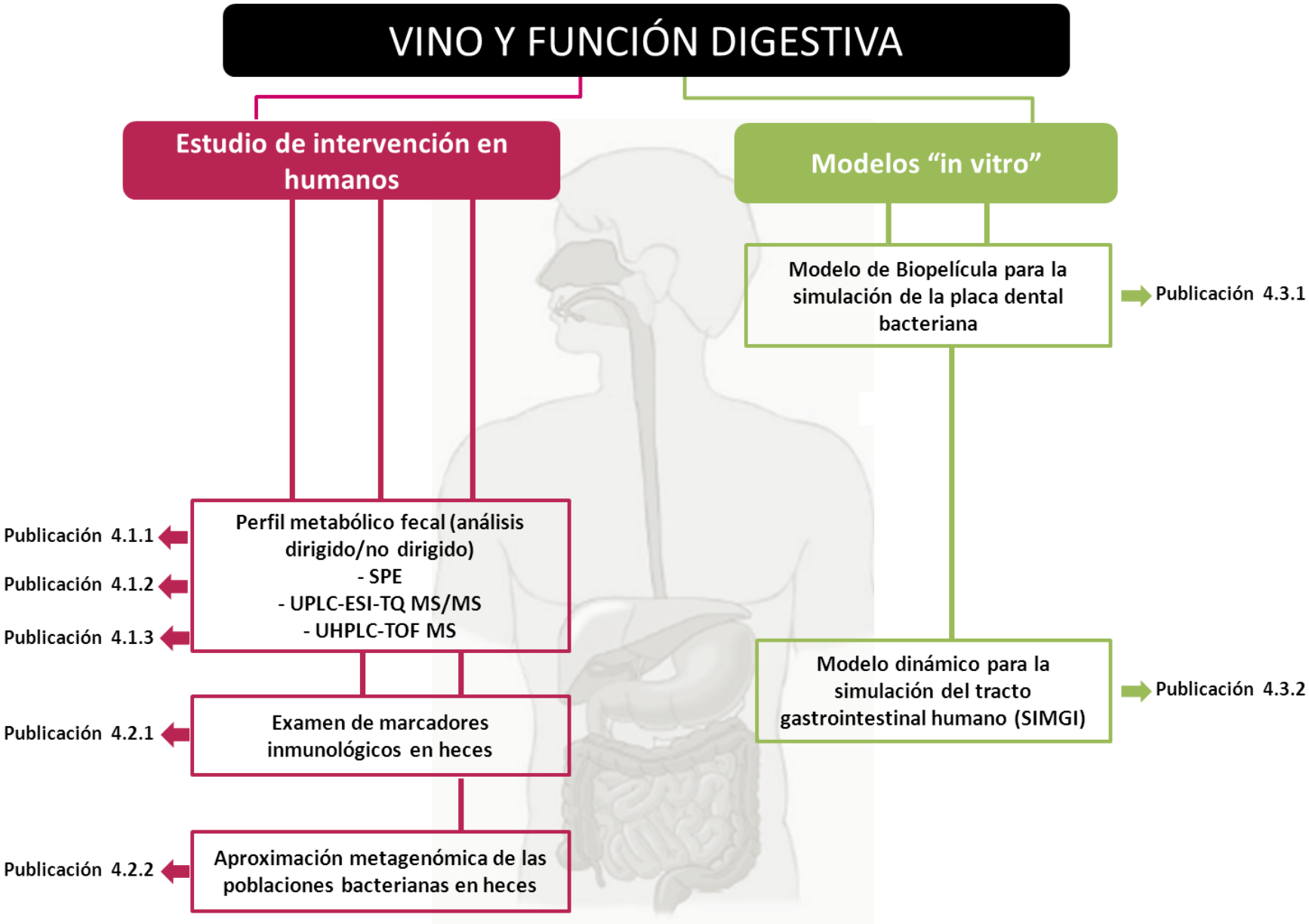

Figura 14. Esquema general del plan de trabajo desarrollado en la presente memoria.

MATERIALES Y MÉTODOS

4. MATERIALES Y MÉTODOS

4.1. Estudio de intervención en humanos con un consumo regular y moderado de vino tinto

4.1.1. Diseño y planificación del estudio

Se diseñó y organizó un estudio de intervención en humanos (**Figura 15**) que incluyó 43 voluntarios sanos, 34 casos y 9 controles, y consistente en los siguientes periodos:

a) Un período de lavado de 2 semanas de duración durante el cual los voluntarios siguieron su dieta habitual pero restringiendo el consumo de alimentos ricos en polifenoles como vino, cerveza, té, café, cacao y frutos rojos. También se restringió el consumo de bebidas alcohólicas.

b) Un periodo de consumo de vino de 4 semanas de duración durante el cual los voluntarios tomaron diariamente 250 mL de vino tinto repartidos en dos tomas (comida y cena) de 125 mL cada una (consumo equivalente a una dosis de 800-1000 mg de polifenoles totales por día), manteniéndose también durante este periodo la restricción del consumo de otros alimentos ricos en polifenoles y de bebidas alcohólicas.

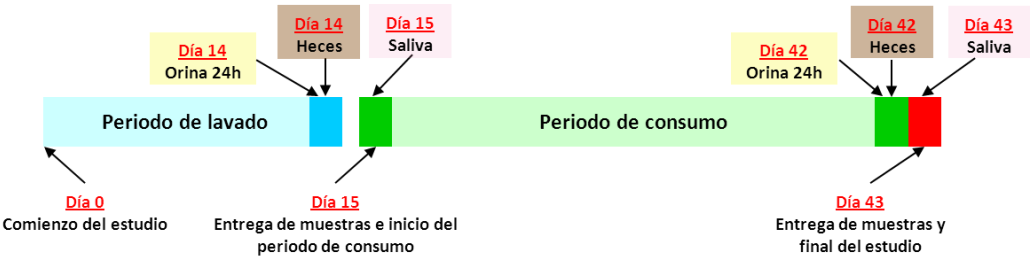

Figura 15. Esquema del diseño del estudio de intervención en humanos.

El vino utilizado en este estudio fue un tinto joven de la variedad Pinot Noir de la vendimia 2010, rico en polifenoles (1758 mg equivalentes de ácido gálico/L), y elaborado por la empresa Miguel Torres S.A. según las prácticas enológicas habituales de la bodega. Su contenido en antocianos totales era 447 mg de malvidin-3-*O*-glucosido/L y en catequinas totales 1612 mg de (+)-catequina/L. La capacidad antioxidante del vino, medida mediante el test ORAC (Dávalos y col., 2004), fue de 35,5 mM Trolox/L, ligeramente superior a los valores encontrados por Landraut y col. (2001) en otros vinos tintos monovarietales (9,6 mM-29,9 mM Trolox/L).

4.1.2. Reclutamiento de voluntarios

Se seleccionó una población homogénea en edad, y sin enfermedades de base relevantes. Para ello, se incluyeron 43 individuos sanos (34 casos y 9 controles), no fumadores, con una edad comprendida entre 20-65 años, y de ambos sexos (23 mujeres y 20 hombres). Como criterio de inclusión, los participantes no debían haber recibido antibióticos u otro tratamiento médico relevante por lo menos durante los 6 meses previos al comienzo del estudio, ni padecer enfermedades o trastornos digestivos. Adicionalmente, no debían usar antisépticos orales durante los 30 días previos al estudio, ni debían padecer ninguna enfermedad sistémica que pudiera alterar la cantidad y composición de la placa dental o de la saliva. El protocolo de investigación fue revisado y autorizado por el Comité de Ética del Hospital Ramón y Cajal (Madrid), y todos los participantes dieron su consentimiento para participar en el estudio tras ser debidamente informados.

4.1.3. Ejecución del estudio y toma de muestras

El estudio de intervención se ejecutó según lo diseñado, sin incidencias. Tras el periodo inicial de lavado, de 2 semanas de duración, se colectaron las siguientes muestras: saliva, orina "24h" y heces. Dichas muestras serán consideradas y nombradas de aquí en adelante como *muestras basales o iniciales*. Una vez concluídas las 4 semanas de consumo moderado de vino, se colectaron de nuevo muestras de saliva, orina "24h" y heces (*muestras finales*). Los 9 sujetos control siguieron las mismas pautas que los voluntarios, con la única excepción que no ingirieron el vino durante las 4 semanas del periodo de intervención. El procedimiento de recogida de las muestras, tanto para los voluntarios que consumieron vino como para el grupo control, fue el siguiente:

- Saliva: el día de la recogida, al levantarse, sin lavarse los dientes y antes de beber o ingerir ningún tipo de alimento, los voluntarios debían llenar su boca con 15 mL de agua, enjuagarse vigorosamente la boca durante 30 segundos, y vaciar el contenido en un tubo falcon estéril.
- Orina "24h": los últimos días de los periodos de lavado y de intervención, el voluntario debía recoger, en un recipiente de 2 L estéril, la primera orina de la mañana y toda la orina durante las 24 horas siguientes, finalizando la recogida la mañana del día de la entrega de las muestras.

- Heces: Las muestras de heces debían ser recogidas los últimos días de cada periodo, tanto el de lavado (*muestras basales o iniciales*) como el de intervención (*muestras finales*) en un recipiente esteril.

4.1.4. Preparación de las muestras biológicas

Las muestras de saliva, colectadas en tubos falcon de 15 mL estériles, se homogeneizaron y se centrifugaron a 10000 rpm, 10 min, 4°C. Tras la centrifugación, se desechó el sobrenadante y se congelaron los pellets a -80°C, para su análisis posterior por PCR-DGGE (Barroso, 2011) (resultados no mostrados en la presente memoria).

Las muestras de orina "24h", colectadas en recipientes de 2 L estériles, se homogeneizaron y acidificaron con ácido clorhídrico hasta una concentración final de 0,2 M. Para ello, una vez homogenizado el contenido del envase de 2 L, se separaron 50 mL de orina en un tubo falcon de 50 mL, y se añadieron 0,87 mL de HCl comercial (37 %, densidad = 1,178 g/mL, VWR BDH Prolabo). Posteriormente, se tomaron 15 alícuotas en tubos eppendorf de 2 mL que se congelaron a -80 °C.

Las muestras de heces, colectadas en recipientes estériles, fueron congeladas a -80 °C hasta su posterior utilización. Para el análisis dirigido y no dirigido de metabolitos fecales, se pesaron 4 g de heces en tubos falcon estériles de 50 mL; se añadieron 40 mL de solución salina estéril y la mezcla se agitó con el vortex hasta su completa homogeneización. Posteriormente, se centrifugó (10 min, 10000 rpm, 4 °C) y se traspasó el sobrenadante a un tubo falcon nuevo, el cual se volvió a centrifugar 1 o 2 veces más hasta la ausencia de pellet. Finalmente, el sobrenadante se filtró con una unidad de filtrado *Stericup® Express™ PLUS 0.22 µm* de Millipore (Billerica, MA, USA) con el fin de obtener una solución fecal limpia y estéril. Para el análisis inmuno-enzimático de marcadores inmunes, se pesaron 100 mg de heces en un tubo eppendorf de 1.5 mL; se añadió 1 mL de tampón fosfato salino (PBS) y se agitó en el vortex hasta su homogenización. Posteriormente, se centrifugó (15 min, 14000 rpm, 4 °C) y se recogió el sobrenadante para su análisis.

4.2. Análisis de metabolitos en muestras fecales

4.2.1. Análisis dirigido de compuestos fenólicos en muestras fecales

4.2.1.1. Extracción en fase sólida (SPE) como paso previo al análisis por UPLC-ESI-TQ MS/MS

Se utilizó un sistema Vacuumm Manifold de extracción a vacío con un rack de 12 posiciones (Teknokroma, Barcelona, España). En todos los experimentos se siguió el protocolo de extracción recomendado por el fabricante de los cartuchos SPE, manteniendo la presión entre 7 y 8 inches de Hg. El protocolo de extracción consistió en los siguientes pasos:

1) **Acondicionamiento** con el fin de activar las moléculas del adsorbente y favorecer su interacción con los analitos.
2) **Equilibrado** del relleno antes de la carga de la muestra.
3) **Carga** de la muestra.
4) **Lavado** del cartucho con un disolvente adecuado para la elución de los compuestos no retenidos en el cartucho.
5) **Elución** de los analitos de interés con un disolvente adecuado.

Para comprobar la eficacia de la SPE como técnica preparativa previa, se prepararon dos soluciones patrón o "pool" (mezclas de patrones #1 y #2) que contenían, respectivamente, 6 y 9 compuestos fenólicos estándar suministrados por Sigma Aldrich Chemical Co. (St. Louis, MO), Phytolab (Vestenbergsgreuth, Germany) o Extrasynthese (Genay, France). Para preparar las soluciones patrón, se disolvieron los patrones comerciales en acetonitrilo/agua (1:4, v/v) en concentraciones variables (**Tabla 6**). También se utilizaron dos soluciones fecales, preparadas según procedimiento descrito en el apartado 4.1.4., a partir de las muestras de heces basal y final de un voluntario, escogido al azar, del estudio de intervención en humanos. Todas las soluciones se mantuvieron en refrigeración hasta ser utilizadas en los diferentes experimentos de SPE.

Tabla 6. Composición (µg/mL) de las mezclas de patrones #1 y #2.

Compuesto	Mezcla #1 (µg/mL)	Mezcla #2 (µg/mL)
Ácido 3-(4-hidroxifenil)propiónico	250	-
Ácido gálico	125	125
Procianidina B2	250	250
(-)-Epicatequina	100	100
Ácido fenilpropiónico	500	500
Ácido 4-hidroxibenzoico 2, 3, 5, 6 d_4	250	250
Ácido protocatéquico	-	100
(+)-Catequina	-	100
Ácido cafeico	-	125
Ácido benzoico	-	250

Por otro lado, con el fin de comprobar qué tipo de fase sólida (relleno) era la óptima para la extracción de compuestos fenólicos en muestras fecales, se ensayaron 4 tipos de cartuchos de las marcas Oasis® (Waters, Milford, MA, EE.UU.) y Bond Elut (Agilent Technologies, Waldbronn, Alemania), con rellenos de 3cc-60 mg de diferente composición y características químicas: Bond Elut Plexa™, Oasis® HLB, Oasis® MCX, Oasis® MAX (**Figura 16**).

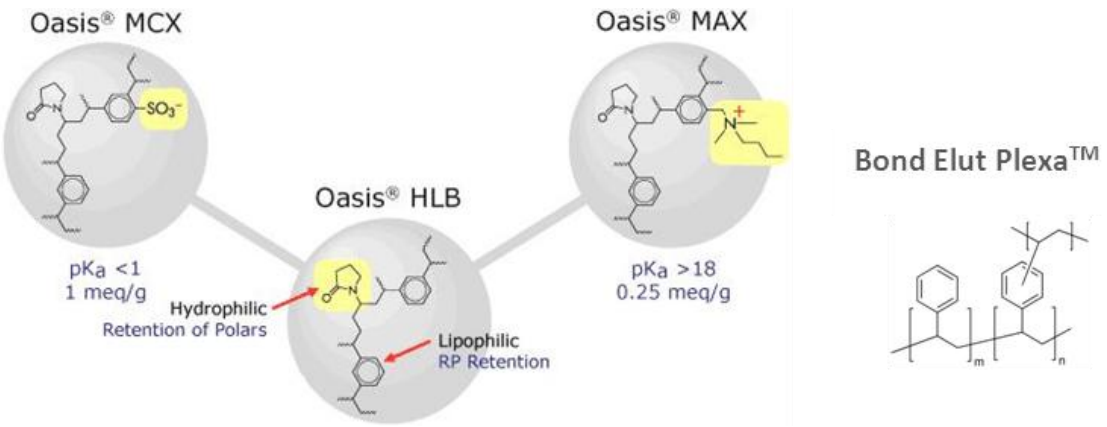

Figura 16. Composición y características químicas de los cartuchos Bond Elut Plexa ™, Oasis® HLB, Oasis® MCX y Oasis® MAX (Adapted from http://www.waters.com).

A continuación, se describen brevemente los experimentos realizados con SPE (**Tabla 7**):

Tabla 7. Objetivo, tipo de cartucho y de muestra utilizados en los experimentos de SPE realizados.

	Experimento 1	Experimento 2	Experimento 3	Experimento 4
Objetivo	Evaluar la idoneidad de 4 tipos de relleno para una mezcla de patrones	Evaluar el efecto de la acidificación de la muestra para una mezcla de patrones	Evaluar la eficacia de la SPE para una mezcla de patrones, con o sin efecto matriz	Evaluar la eficacia de la SPE para una solución fecal
Cartucho	Bond Elut Plexa™ Oasis® HLB Oasis® MCX Oasis® MAX	Oasis® HLB	Oasis® HLB	Oasis® HLB
Muestra	Solución fecal dopada con la mezcla#1: pre-dopada[a] post-dopada[b]	Solución fecal acidificada o no, y dopada con la mezcla#1: pre-dopada[a] post-dopada[b]	Mezcla #2 y solución fecal dopada con la mezcla#2, ambas acidifcadas: pre-dopada[a] post-dopada[b]	Solución fecal acidificada: - Antes SPE - Después SPE [carga simple (2 mL) o carga múltiple (2 mL x 3)]

[a]**pre-dopada**: muestra dopada antes del proceso de SPE
[b]**post-dopada**: muestra dopada después del proceso de SPE

Todas las fracciones obtenidas de los experimentos en las etapas de carga, lavado y elución (≈2mL) se llevaron, con agua MilliQ, a un volumen final de 10 mL antes de su análisis por UPLC-DAD-ESI-TQ MS. Igualmente se analizó el agua fecal sin ser sometida a SPE. Todos los ensayos se realizaron por duplicado. Los porcentajes de recuperación se calcularon por la división de los valores de concentración obtenidos en la muestra pre-dopada respecto a la muestra post-dopada (experimentos 1, 2 y 3) y de la muestra sometida a SPE respecto a la muestra no sometida a SPE (experimento 4).

4.2.1.2. Análisis dirigido de compuestos fenólicos por UPLC-ESI-TQ MS/MS

El análisis se llevó a cabo con un equipo Waters Aquity UPLC (Milford, MA, EE.UU.) constituido por una bomba binaria y un detector ultravioleta-visible de diodos alineados (DAD) modelo eλ y

acoplado, en serie, a un espectrómetro de masas de triple cuadrupolo (TQ MS/MS) con fuente de ionización por electro-nebulización (ESI) (**Figura 17**). La separación cromatográfica se llevó a cabo en una columna Waters® UPLC BEH C_{18} (2,1 x 100 mm; 1,7 µm) a una temperatura de 40 °C y empleando un flujo de 0,5 mL/min.

Figura 17. Equipo Acquity UPLC-ESI-TQ MS/MS empleado.

Se utilizó una solución de patrones puros compuesta por 47 compuestos fenólicos pertenecientes a diferentes familias de ácidos fenólicos (ácidos hidroxifenilpropiónicos, hidroxifenilacéticos, hidroxicinámicos, hidroxibenzoicos, hidroximandélicos) y fenoles simples. También se incluyeron compuestos del grupo de los flavan-3-oles: (+)-catequina, (-)-epicatequina y procianidinas B1 y B2. Los patrones fueron suministrados por Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), Phytolab (Vestenbergsgreuth, Alemania) y Extrasynthese (Genay, Francia). Para llevar a cabo la separación cromatográfica se utilizaron como eluyentes A: 2% ácido acético en agua y B: 2% ácido acético en acetonitrilo. El gradiente de elución se muestra en la **tabla 8**.

Tabla 8. Gradiente de separación cromatográfica utilizado para la separación de metabolitos fenólicos por UPLC.

Tiempo (min)	% Fase A	%Fase B
0	99.9	0.1
1.5-11.17	99.9-83.7	0.1-16.3
11.17-11.5	83.7-81.6	16.3-18.4
11.5-14	81.6	18.4
14-14.1	81.6-0.1	18.4-99.9
14.1-15.5	0.1	99.9
15.5-15.6	99.9	0.1
15.6-18	99.9	0.1

Los parámetros de ionización fueron: voltaje del capilar, 3 kV; fuente de temperatura, 130 °C; temperatura de desolvatación, 400 °C; flujo gas de desolvatación (N_2), 750 L/h; flujo gas (N_2) del cono, 60 L/h. La fuente de ionización se utilizó en modo negativo. Previo a la inyección (2 µL), las soluciones fecales, previamente diluidas, limpias y estériles (apartado 3.1.4), fueron diluidas con acetonitrilo (1:4, v/v, acetonitrilo/solución fecal). Las muestras se analizaron por duplicado.

Para la cuantificación, los datos fueron recogidos en el modo de reacción múltiple (MRM), siguiendo la transición de los iones padre y producto específicos para cada compuesto, y usando curvas de calibración externas (Sánchez-Patán y col., 2011), en el caso de la publicación 5.1.1, o curvas de calibración internas (Jiménez-Girón y col., 2013), para el caso de las demás publicaciones (publicaciones 5.1.2, 5.3.1 y 5.3.2). El análisis de los datos se llevó a cabo utilizando el software Masslynx (Waters, Milford, MA, EE.UU.).

4.2.2. Análisis no dirigido del metaboloma fecal por UHPLC-TOF MS

El análisis metabolómico de las muestras fecales se llevó a cabo en la Plataforma de Metabolómica del Instituto de Investigación en Ciencias de la Alimentación (CIAL) (www.cial.uam-csic.es/metabolomica), utilizando un equipo de ultra alta eficacia (UHPLC system 1290) de Agilent Technologies (Palo Alto, CA, EE.UU.) acoplado a un detector espectrómetro de masas de tiempo de vuelo de alta resolución (Q/TOF MS) (Agilent 6540), con interfase de electrospray (ESI) (Agilent Jet Stream, AJS), y operando en modo negativo (Agilent Technologies, Palo Alto, CA, EE.UU.) (**Figura 18**).

Figura 18. Equipo UHPLC-TOF MS empleado.

Previo a la inyección (2 µL), las soluciones fecales, previamente diluidas, limpias y estériles (apartado 4.1.4), fueron diluidas con acetonitrilo (1:4, v/v, acetonitrilo/solución fecal). Las muestras se inyectaron, por duplicado, en una columna de fase reversa ZORBAX C8 (2.1 × 100 mm, 1.8 µm). La temperatura de la columna se mantuvo a 40 °C. La separación cromatográfica se llevó a cabo utilizando los eluyentes A: 0,01% ácido fórmico en agua y B: 0,01% ácido fórmico en acetonitrilo, siguiendo el gradiente presentado en la **tabla 9**:

Tabla 9. Gradiente de separación cromatográfica utilizado para la separación de metabolitos fenólicos por UHPLC.

Tiempo (min)	% Fase A	%Fase B
0-7	100-70	0-30
7-11	70-0	30-100
11-14	0	100
14-21	100	0

Los parámetros de análisis por masas fueron los siguientes: voltaje de capilar, 4000 V; presión del nebulizador, 40 psi; flujo del gas nebulizador, 10 L/min; temperatura del gas, 350 °C; voltaje de cono, 45 V; voltaje de fragmentación, 110 V. El registro de la masa exacta se realizó en el rango desde 50 a 1000 m/z, y se usó el software MassHunter (Agilent Technologies, Palo Alto, CA, EE.UU.) para la adquisición de espectros, el análisis cualitativo y semi-cuantitativo, y para la identificación de metabolitos.

La calibración interna del espectrómetro de masas Q/TOF se llevó a cabo automáticamente utilizando una solución de estándares de referencia compuesta por: 9 µM de purina ($[C5H5N4]^-$, 119,03632 m/z) y 0.5 µM de hexakis(1H,1H,3H-tetrafluoropropoxi)fosfaceno ($[C18H19O6N3P3F24]^-$, 921,0031 m/z) en acetonitrilo-agua (95:5, v/v). La calibración externa del TOF MS se llevó a cabo utilizando una mezcla comercial de compuestos suministrados por el fabricante del equipo, cuyos valores de m/z fueron: 301,998139, 601,978977, 103,988109, 1333,968947, 1633,949786, 1933,930624, 2233,911463, 2533,892301, 2833,873139.

Los datos fueron procesados y convertidos al formato mzXML mediante el programa Trapper (Versión 4.3.0). La detección e integración de los picos se llevó a cabo utilizando el software MZmine (versión 2.7.2). Para la pre-selección de los datos obtenidos, se establecieron las siguientes condiciones

de exclusión: (i) picos con intensidad 3 veces menor al blanco. (ii) picos no encontrados en, al menos, el 75% de las muestras pertenecientes al mismo grupo de voluntarios. (i.e. antes y después del consumo de vino) o con una gran variabilidad dentro del mismo grupo.

4.3. Análisis relacionados con la función intestinal

4.3.1. Análisis inmuno-enzimático de marcadores inmunes en muestras fecales

4.3.1.1. Ensayo multiplex para la cuantificación de inmunoglobulinas y citoquinas

La detección y cuantificación de inmunoglobulinas (Ig): IgE, IgM, IgG_1, IgG_2, IgG_3, IgG_4; y citoquinas, incluyendo interleuquinas (IL): 1β, 2, 4, 5, 6, 7, 8, 10, 12 (p70), 13, y 17, interferón-gamma (IFN-γ), factor de necrosis tumoral-α (TNF-α), oncogén regulador de crecimiento-α (Gro-α), proteína quimiotáctica de monocitos-1 (MCP-1), proteína inflamatoria de macrófagos-1β (MIP-1β), factor estimulante de colonias de granulocitos (G-CSF) y factor estimulante de colonias de granulocitos y monocitos (GM-CSF), se llevaron a cabo mediante dos sistemas multiplex de microesferas magnéticas en suspensión (Bio-Plex Pro Human Cytokine 17-Plex Panel y Bio-Plex Pro Human Isotyping Panel) de BioRad laboratories (Hercules, CA, EE.UU.), los cuáles permiten la cuantificación simultánea de 17 citoquinas y 6 inmunoglobulinas, respectivamente. La **tabla 10** muestra la composición en citoquinas e inmunoglobulinas diana:

Tabla 10. Composición en inmunoglobulinas y citoquinas diana de los sistemas Bio-Plex utilizados.

Bio-Plex Pro Human Cytokine 17-Plex Panel		Bio-Plex Pro Human Isotyping Panel
G-CSF	IL-8	IgE
GM-CSF	IL-10	IgM
IFN-γ	IL-12 (p70)	IgG_1
IL-1ß	IL-13	IgG_1
IL-2	IL-17	IgG_1
IL-4	MCP-1	IgG_1
IL-5	MIP-1ß	
IL-6	TNF-α	
IL-7		

El análisis inmuno-enzimático de inmunoglobulinas y citoquinas se llevó a cabo siguiendo el protocolo especificado por el fabricante, el cual se describe a continuación:

Una vez diluidos los estándares suministrados por el fabricante, y preparadas las muestras (apartado 4.1.4), se procedió a humectar la placa de 96 pocillos mediante la adición de la solución tampón suministrada por el fabricante, y su eliminación mediante filtración a vacío utilizando un equipo Bio-Plex Pro II Wash Station (Bio-Rad Laboratories, Hercules, CA, EE.UU.). A continuación, en cada pocillo se adicionaron 50 µL de las microesferas magnéticas, mezcladas previamente y marcadas con los anticuerpos específicos. Se realizaron 2 lavados mediante filtración a vacío de los pocillos con 100 µL de tampón de lavado, y seguidamente se añadieron 50 µL de los estándares y controles (por triplicado), y de las muestras (por duplicado). Las placas se incubaron a temperatura ambiente con agitación suave durante 30 minutos (en el caso del análisis de citoquinas) o 1 hora (en el caso del análisis de inmunoglobulinas). Tras la incubación, se realizaron 3 nuevos lavados con tampón de lavado y se añadieron 25 µL del anticuerpo de detección (marcado con biotina) en cada pocillo. Las placas se incubaron a temperatura ambiente con agitación suave durante 30 minutos (formación de un "sándwich" de anticuerpos alrededor de la citoquina o inmunoglobulina diana). Tras 3 nuevos lavados de los pocillos con tampón de lavado, se adicionaron 50 µL de la solución de estreptavidina-ficoeritrina (SAPE) en cada pocillo y se incubó la placa a temperatura ambiente con agitación suave durante 10 minutos para permitir que la SAPE se uniera al anticuerpo de detección en la superficie de la microesfera. Finalmente, los pocillos se lavaron 3 veces con tampón de lavado y se resuspendieron las en 125 µL de tampón de ensayo para proceder a la cuantificación.

Para la cuantificación de los marcadores inmunológicos, se utilizó un instrumento Bioplex 200 (Bio-Rad Laboratories, Hercules, CA, EE.UU.) acoplado a un sistema Luminex® (Austin, TX, EEUU), basado en la citometría de flujo. La determinación de las muestras se realizó por duplicado. Las curvas de calibrado de cada marcador inmune se realizaron con el software Bio-Plex Manager 6.0 (Bio- Rad Laboratories, Hercules, CA, EE.UU.). El coeficiente de variación para las curvas estándar entre placas fue inferior al 10 % en todos los casos. A partir de las curvas de calibrado, se calculó la concentración de cada inmunoglobulina/citoquina en las muestras.

4.3.1.2. Ensayo ELISA para la cuantificación de inmunoglobulina A (IgA)

La concentración de inmunoglubulina A (IgA) en las muestras fecales se determinó utilizando un kit ELISA (Cusabio Biotech Co., LTD, Wuhan, China), siguiendo el protocolo especificado por el fabricante, el cual se describe a continuación:

Una vez diluido el estándar suministrado por el fabricante, y preparadas las muestras (apartado 3.1.4), se añadieron 100 µL de cada uno de ellos (estándares por triplicado y muestras por duplicado) en sus pocillos correspondientes, y se incubó la placa a 37 °C durante 2 h. A continuación, se eliminó el contenido de los pocillos mediante filtración a vacío (Bio-Plex Pro II Wash Station) y se lavaron los pocillos 3 veces consecutivas durante 2 minutos con 200 µL de tampón de lavado. Se añadieron 100 µL del anticuerpo específico para IgA conjugado con la enzima peroxidasa de rábano (HRP) y se incubaron las placas a 37 °C durante 1 h. Una vez finalizada la incubación, se realizaron 5 lavados consecutivos de los pocillos con tampón de lavado y se añadieron 90 µL de sustrato enzimático cromogénico TMB (3,3,5,5-tetrametilbencidina). A continuación se incubó la placa a 37 °C durante 20 minutos, en oscuridad, y se añadieron 50 µL de solución de parada para detener la reacción. Inmediatamente, se midió la densidad óptica durante 5 minutos a 450 nm en un espectrofotómetro de placas. Además, se realizó una medida a 540 nm, con el fin de corregir el efecto de las posibles imperfecciones de la placa. Los valores de concentración de IgA en las muestras fueron calculados mediante la extrapolación de los valores de absorbancia en la curva de calibrado construida con los valores de las soluciones estándar.

4.3.1.3. Ensayo ELISA para la cuantificación de calprotectina

La concentración de calprotectina en las aguas fecales se determinó utilizando un kit ELISA (Calpro, Lysaker, Noruega), siguiendo el protocolo especificado por el fabricante, similar al utilizado para la cuantificación de IgA, en el apartado anterior. La curva de calibrado se obtuvo a partir de triplicados de cada concentración de estándar y se ajustó a un modelo de curva de 4 parámetros de acuerdo a las instrucciones del fabricante.

4.3.2. Análisis metagenómico de poblaciones bacterianas en muestras fecales

Previo a su análisis, las muestras fecales se descongelaron a temperatura ambiente. Se pesaron 0,1 g en un tubo eppendorf y se añadieron 0,5 mL de tampón de extracción (200 mM de Tris-HCl, pH 7,5, 0,5 % de SDS, EDTA 25 mM, NaCl 250 mM, 20 mg/mL de lisozima, 5 mg/mL de lisostafina) y 0,3 mL de acetato de sodio 3 M. Tras la homogenización de la mezcla, se llevó a cabo una lisis mecánica con perlas de zirconia de 0,1 mm de diámetro, usando un disruptor FastPrep (Qbiogene, Irvine, CA, EE.UU.) a una velocidad de 6,0 m/s durante 30 s; este tratamiento se realizó tres veces, manteniendo las muestras en hielo durante 1 minuto entre tratamientos. A continuación, se añadieron 0,1 mg/mL de proteinasa K de Sigma-Aldrich (St. Louis, MO, EE.UU.), y se incubó a 37 °C durante 30 min. Tras la incubación, se añadieron 0,1 mL de NaCl 1,5 M, se mezcló y se incubó la mezcla a temperatura ambiente durante 5 min; por último, se centrifugó a 14000 rpm para sedimentar los restos celulares insolubles. El sobrenadante obtenido se transfirió a un nuevo tubo eppendorf y se extrajo dos veces con un volumen igual de fenol/cloroformo/isoamilo-alcohol (25:24:1) (Sigma-Aldrich, St. Louis, MO, EE.UU.). El ADN se precipitó mediante la adición de 0,6 volúmenes de isopropanol (Sigma-Aldrich, St. Louis, MO, EE.UU.) e incubando a -20 °C durante 1 h. El ADN ya sedimentado se lavó con etanol al 70%, se dejó secar al aire, y finalmente se resuspendió en tampón TE (Tris-EDTA). Se utilizó un kit de extracción de gel QIAEX® II (QIAGEN, Hilden, Alemania) para purificar y concentrar el ADN. El rendimiento de ADN se midió utilizando un espectrofotómetro UV NanoDrop® ND-1000 (Tecnología Nano-Drop, Wilmington, DE, EE.UU.).

La secuenciación masiva en paralelo de amplicones del 16S rDNA en las muestras se llevó a cabo empleando la Plataforma Illumina MiSeq Personal Sequencer (Illumina Inc., San Diego, CA, EE.UU.) ubicada en el Parque Científico de Madrid (Campus de Cantoblanco, Universidad Autónoma de Madrid, Madrid). Las muestras de ADN se amplificaron mediante PCR usando los cebadores directos 27F-Degl (5'GTTYGATYMTGGCTCAG-3') en combinación con una mezcla equimolar de dos cebadores inversos, 338R-I (5'-GCWGCCTCCCGTAGGAGT-3') y 338R-II (5'-GCWGCCACCCGTAGGTGT-3'), generando amplicones de aproximadamente 345 pb de las regiones hipervariables V1 y V2 del gen codificante del 16S rDNA. La amplificación por PCR se realizó utilizando el Fast Start High Fidelity PCR System dNTP Pack (Roche, Mannheim, Alemania) en un volumen total de 25 µL que contenía 2,5 µL de tampón de reacción 10X, 1,25 unidades de Fast Start High Fidelity Enzyme Blend (Roche, Mannheim, Alemania),

0,4 M de cada cebador, y 10 ng de ADN molde. Las condiciones del proceso fueron las siguientes: una desnaturalización inicial a 95 °C durante 2 minutos, y 35 ciclos a 95 °C durante 30 s, 54 °C durante 30 s, y 72 °C durante 30 s, con una extensión final a 72 °C durante 5 minutos. Tras la amplificación, se utilizaron 2 µL del producto de PCR para la comprobación mediante electroforesis con gel de agarosa.

Los códigos de barras utilizados para la secuenciación fueron añadidos a los extremos 3 'y 5' de los amplicones en una segunda PCR para permitir la separación de las secuencias directa e inversa. Todos los "primer" fueron sintetizados por Isogen Life Sciences (Castelldefels, España). Los productos de PCR se agruparon en concentraciones equimolares de ADN y se corrieron en un gel de agarosa preparativo. La banda del tamaño correcto se escindió, se purificó usando un kit de extracción de gel QIAEX II (QIAGEN, Hilden, Alemania) y se cuantificó con PicoGreen (BMG Labtech, Jena, Alemania).

4.4. Sistemas de simulación del tracto buco-gastrointestinal

4.4.1. Modelo de biopelícula oral para la simulación de la placa supragingival dental

Se utilizó un modelo de biopelícula para la simulación de la placa supragingival humana consistente en las siguientes especies bacterianas: *Actinomices oris* OMZ745, *Velionella dispar* OMZ493, *Fusobacterium nucleatum* OMZ598, *Streptococcus* oralis OMZ607 y *Streptococcus mutans* OMZ 918. Las bacterias utilizadas se obtuvieron de la colección del Instituto de Biología Oral de la Universidad de Zürich.

Las bacterias se sembraron en placas de agar Columbia Sangre, que se incubaron a 37°C durante 96h en condiciones anaerobias. Después de este tiempo, las cepas fueron transferidas a tubos falcon estériles con 9 mL de medio de cultivo mFUM [medio fluido universal (FUM), modificado mediante la adición de tampón de Sorensen´s (67 mM)] con 0,3 % de glucosa. Para el crecimiento óptimo de *Velionella dispar* OMZ493 también se añadió un 1 % de lactato de sodio al medio mFUM. Los tubos se incubaron durante toda la noche a 37 °C en anaerobiosis. Tras la incubación "overnight", 200 µL de cada cultivo bacteriano se transfirieron de nuevo a un tubo falcon con 5 mL de medio mFUM fresco y se incubaron a 37 °C en anaerobiosis durante un máximo de 7 h. A continuación, se ajustaron las densidades ópticas de los 5 cultivos bacterianos, con el fin de obtener cultivos en la fase de

crecimiento exponencial (aproximadamente 10^7 CFU/mL) y se preparó un inóculo mezclando 200 µL de cada cultivo bacteriano (volumen final del inóculo: 1 mL).

Como fase previa a la formación de las biopelículas, se recogió y procesó saliva de cinco voluntarios sanos, siguiendo el protocolo de Guggenheim y col. (2004). De forma resumida, la saliva no estimulada se recogió durante 1 h por la mañana, durante varios días, y al menos 1,5 h después de comer, beber, o lavarse los dientes. La saliva se recolectó en tubos falcon de 50 mL estériles, conservados en hielo durante la recogida, y posteriormente congelados a -20 °C. Una vez alcanzada una cantidad mínima de 500 mL, se descongeló la saliva recogida, se homogeneizó y se centrifugó durante 30 min a 4 °C y 14000 rpm; el sobrenadante se pasteurizó (60 °C, 30 min), se centrifugó nuevamente en tubos falcon estériles y se almacenó en tubos falcon estériles de 50 mL a -80 °C hasta su utilización. Como control del proceso, se sembró la saliva en placas de agar Columbia con sangre para verificar que no hubiera crecimiento bacteriano (72 h, 37 °C, anaerobiosis). Se diluyó la saliva mezclando partes iguales de la saliva estéril con una solución estéril de agua y NaCl fisiológica (75:25, v/v) para obtener una solución de saliva estéril.

La formación de la biopelícula oral se llevó a cabo en discos de hidroxiapatita de 9 mm de diámetro (Clarkson Chromatography Products, Inc., South Williams-port, PA, EE.UU.) alojados en placas de 24 pocillos, y siguiendo los protocolos de Guggenheim y col. (2004) y Thurnheer y col. (2006) (**Figura 19**).

Los discos se pre-condicionaron con 800 µL de la solución saliva estéril durante 4 horas a temperatura ambiente y con agitación suave (95 rpm), con el fin de promover la formación de una película de proteínas de origen salival. Para iniciar la formación de la biopelícula, los discos se incubaron durante 45 minutos con 1,6 mL de una mezcla de la solución de saliva estéril y mFUM (30:70; v/v) y 200 µL del inóculo bacteriano (5 especies). La concentración de hidratos de carbono en el mFUM fue de 0,3 % de glucosa (w/v), en los primeros pasos (16 h) de formación de la biopelícula, y de 0,15 % de glucosa + 0,15 % de sacarosa en los siguientes pasos. Posteriormente, los discos se sometieron a tres lavados consecutivos con 1,6 mL de solución salina al 0,9 % durante 1 min, con el fin de eliminar las bacterias no agregadas a la biopelícula. A continuación, se incubaron en solución de saliva estéril pre-condicionada (37 °C, 45min, anaerobiosis) durante 16,5 h a 37 °C en condiciones

anaeróbicas (biopelícula formada). Una vez formada la biopelícula, se llevaron a cabo dos ensayos que se describen a continuación (**Figura 19**):

<u>Ensayo 1</u>: Actividad antimicrobiana del vino y extractos de origen enológico frente a las bacterias de la biopelícula oral

Para ensayar el efecto antimicrobiano del vino y algunos componentes inherentes al vino se ensayaron las siguientes soluciones:

- **Vino tinto** (descrito en el apartado 4.1.1)
- **Vino tinto desalcoholizado** mediante evaporación a baja temperatura con un rotavapor R-210 (Buchi, Flawil, Suiza) y reconstituido con agua hasta el volumen inicial.
- **Solución de extracto de vino en agua** (1,6 g/L)
- **Solución de extracto de vino + extracto de pepita de uva** (2,5 g/L)
- **Solución de extracto de vino + extracto de levaduras secas inactivas rico en manoproteínas** (0,4 g/L)
- **Solución de extracto de vino + extracto de levaduras secas inactivas rico en péptidos** (0,4 g/L)

Para la preparación de las soluciones de extractos se utilizaron: un extracto de vino (Provinols®) rico en polifenoles (474 mg de equivalentes de ácido gálico/g), suministrado amablemente por Safic-Alcan Especialidades S.A.U. (Barcelona, Spain); un extracto de pepita de uva (Vitaflavan®) rico en polifenoles (629 mg de equivalentes de ácido gálico/g), especialmente en flavan-3-oles, suministrado amablemente por Les Dérivés Resiniques & Térpeniques S.A. (Francia); y dos extractos comerciales de levaduras secas inactivas, cuya composición ha sido caracterizada previamente por el grupo de investigación Biotecnología Enológica Aplicada (CIAL, CSIC-UAM), ricas en manoproteínas y péptidos, componentes de la matriz del vino.

Tras la formación de las biopelículas, los discos se mantuvieron durante todo el experimento (7 días) en pocillos con solución de saliva estéril a 37 °C y en anaerobiosis, la cual fue re-cambiada todos los días. Dos veces al día, con 7 horas de diferencia, se extrajeron los discos y se sumergieron en las diferentes soluciones (1 mL) durante 2 min con agitación suave. Antes de cada inmersión, se incubaron

los discos durante 45 min en una mezcla de solución de saliva estéril y medio mFUM (30:70, v/v), conteniendo un 0,15 % de glucosa y un 0,15 % de sacarosa, en condiciones anaerobias; posteriormente a cada inmersión, los discos se lavaron mediante su inmersión en solución de saliva estéril, con el fin de eliminar los posibles restos de las soluciones ensayadas.

En este ensayo, se utilizó agua destilada estéril como control negativo antimicrobiano, y una solución de clorhexidina-gluconato (Sigma Aldrich, Steinheim, Alemania) al 0,2 % en agua, como control positivo. Los experimentos se llevaron a cabo por triplicado.

<u>Ensayo 2</u>: Metabolismo de los compuestos fenólicos del vino por parte de la biopelícula oral: Análisis dirigido de compuestos fenólicos (UPLC-ESI-TQ MS/MS)

Para el llevar a cabo este experimento se procedió a incubar lo discos con las biopelículas ya formados en medio de crecimiento mFUM al que se añadió la solución de extracto de vino a una concentración de 1,6 g/L, en ausencia y presencia de extracto de pepita de uva (10 g/L), durante 24 h, a 37 °C y en condiciones anaerobias. A las 0, 2, 4, 8 y 24 h se recogió parte del sobrenadante y se filtró por un filtro de 0.22 µm. Se analizaron los sobrenadantes por UPLC-ESI-TQ MS/MS siguiendo el método de análisis de metabolitos fenólicos, descrito en el apartado 3.2.1.2.

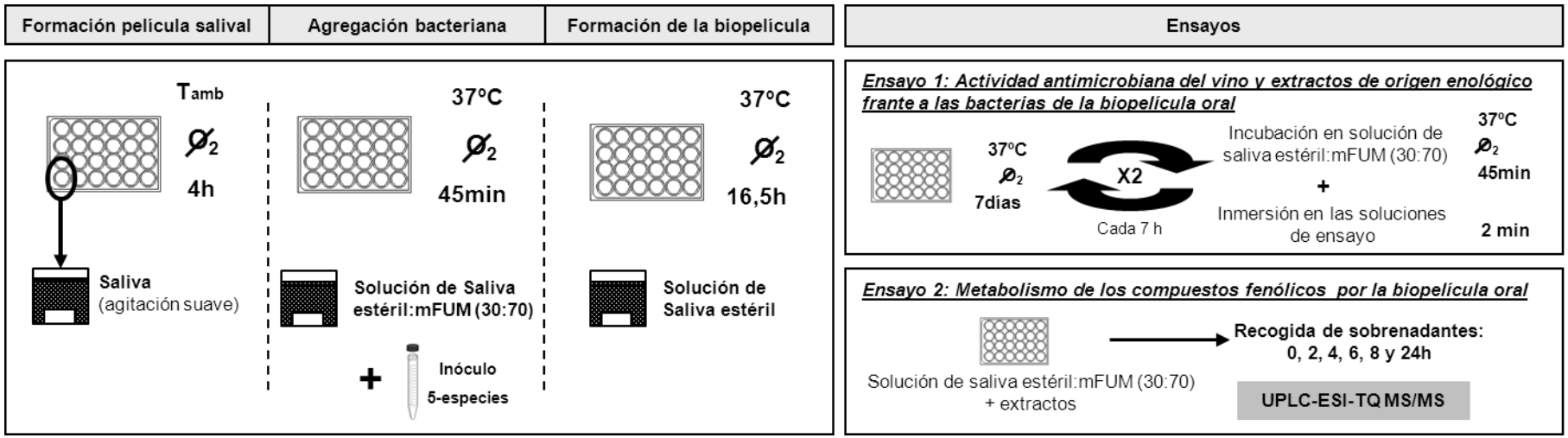

Figura 19. Diagrama de la formación de la biopelícula oral de 5 especies bacterianas y los ensayos realizados.

4.4.1.1. Recuento bacteriano en medios selectivos

Al finalizar los 7 días de ensayo, se recuperaron las biopelículas formadas (**Figura 20**) mediante su raspado con un utensilio odontológico (sonda dental) y su re-suspensión en una solución de NaCl al 0,9%. La viabilidad celular de la suspensión obtenida se evaluó mediante un kit Live/Dead® BacLight™ de viabilidad bacteriana (Invitrogen, Zug, Suiza).

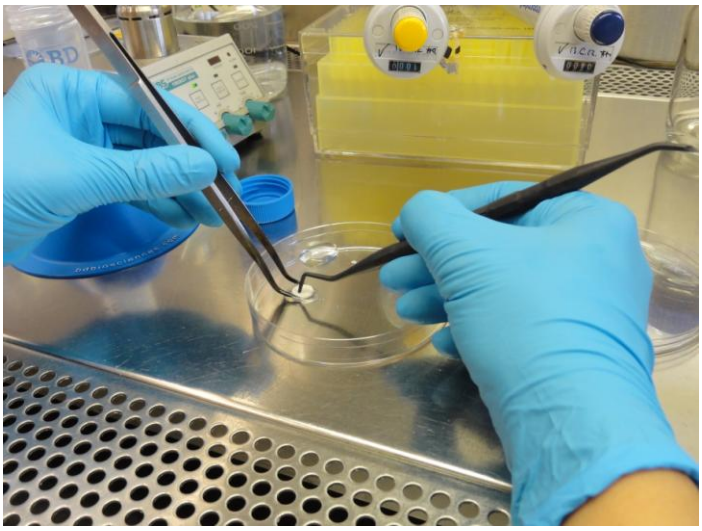

Figura 20. Recuperación de la biopelícula oral mediante su raspado con sonda dental.

Una vez comprobada la viabilidad y estimada la concentración de células vivas, las biopelículas recuperadas, en suspensión, fueron diluidas de forma seriada. El número de unidades formadoras de colonias (UFC/mL) totales, y de cada especie bacteriana, se cuantificaron mediante el recuento de colonias tras la siembra directa de las diluciones en placas de medios selectivos (Agar Mitis Salivarius para *Streptococcus oralis* y *Streptococcus mutans*; Agar Fastidioso Anaerobio para *Fusobacterium nucleatum*), y no selectivos (Agar Columbia sangre para *Actinomyces oris*, *Veillonella dispar* y para el recuento total de bacterias), a 37 °C en anaerobiosis.

4.4.1.2. Análisis de viabilidad bacteriana por microscopía láser confocal de barrido (CLSM)

La microscopía láser confocal de barrido (CLSM) se utilizó para comprobar la viabilidad bacteriana en las biopelículas. Para ello, las biopelículas tratadas se tiñeron utilizando un kit Dead/Live®BacLight™

de viabilidad bacteriana (Invitrogen, Zug, Suiza), de acuerdo con las instrucciones del fabricante. Tras 20 min de tinción, se aspiró suavemente el exceso de tinte, se añadieron 20 µL de Mowiol y se incubaron en oscuridad, a temperatura ambiente y durante 20 min, antes de su examen en el microscopio. Las biopelículas teñidas se examinaron por CLSM en posiciones seleccionadas aleatoriamente utilizando un microscopio Leica SP5 (Leica Microsystems, Heidelberg, Alemania) con un objetivo de 10X/0.8 y 63X/1.4 y utilizando aceite de inmersión. Se utilizó un láser de 488 nm de excitación y filtros de emisión a 530 nm para células vivas y de 561 nm y 640 nm para células muertas. Los datos fueron procesados utilizando el software Imaris 7.2.2 (Bitplane AG., Zürich, Suiza).

4.4.1.3. Análisis dirigido de compuestos fenólicos por UPLC-ESI-TQ MS/MS

El análisis se llevó a cabo con un equipo Waters Aquity UPLC (Milford, MA, EE.UU.) acoplado a un espectrómetro de masas de triple cuadrupolo (TQ MS/MS), con fuente de ionización por electro-nebulización (ESI), y siguiendo el método descrito en el apartado 4.2.1.2.

4.4.2. Modelo de simulación dinámica del tracto gastrointestinal (SIMGI)

Para la simulación de la simulación de la ingesta de vino, se utilizó un simulador dinámico del tracto gastrointestinal (SIMGI), ubicado en el Instituto de Investigación en Ciencias de la Alimentación (CIAL,CSIC-UAM), compuesto por cinco compartimentos, incluyendo estómago [**Figura 21 (A)**], intestino delgado [**Figura 21 (D)**] y colon (ascendente, transverso y descendente) [**Figura 21 (E, F y G, respectivamente)**]. El SIMGI se controla por un autómata, y dispone de cajas controladoras (**B, C**) y bombas (**H**) que regulan el flujo de líquidos (ácido, base, jugo gástrico, alimento) y de gases (nitrógeno). La temperatura se mantiene a 37 °C y el pH a 5,6 ± 0,2, 6,3 ± 0,2 y 6,8 ± 0,2, en el colon ascendente, transverso y descendente, respectivamente. Todos los compartimentos se mantienen en condiciones anaeróbicas por flujo continuo de nitrógeno.

El sistema se alimenta de forma continua con un medio nutritivo, y, de forma programada, se incorpora ácido clorhídrico, para simular las condiciones ácidas del contenido procedente del estómago, y jugo pancreático.

Figura 21. Simulador dinámico del tracto gastrointestinal (SIMGI). (A) Estómago; (B,C) Cajas controladoras; (D) Intestino delgado; (E,F,G) Colon ascendente, transverso y descendente, respectivamente; (H) Bombas.

El medio nutritivo se preparó mediante la disolución de arabinogalactano (1 g/L), pectina de manzana (2 g/L), xilano (1 g/L), almidón de patata (3 g/L), glucosa (0,4 g/L), extracto de levadura (3 g/L), peptona (1 g/L), mucina (4 g/L) y L-cisteína (0,5 g/L) en agua destilada y posterior esterilización a 121 °C durante 15 min. Para simular el medio procedente del estómago, el medio nutritivo se acidificó con HCl al 37 % hasta alcanzar un pH de 2,0. Para simular el jugo pancreático, se preparó una solución de 12 g/L de $NaHCO_3$ en agua, 6 g/L de OX-GALL (bilis de buey deshidratado) y 0,9 g/L de pancreatina de porcino.

Los fermentadores relativos al intestino delgado y colon (ascendente, transverso y descendente) se inocularon con una suspensión fecal (20%, w/v) preparada a partir de heces frescas procedentes de voluntarios sanos (V1y V2). Las muestras fecales se diluyeron en un tampón de fosfato de sodio (0,1 M, pH 7) con tioglicolato de sodio 1 g/L. Se realizaron dos experimentos, utilizando las muestras del V1 o V2 para la inoculación del sistema, respectivamente.

Inicialmente se procedió a la estabilización del sistema y de las poblaciones bacterianas intestinales siguiendo el método de operación del sistema, previamente optimizado por Barroso y col. (2014). El sistema se mantuvo en funcionamiento, bajo todas las condiciones programadas, durante dos semanas

tras la inoculación con la suspensión fecal (voluntario V1). Una vez estabilizado, el sistema se alimentó con 225 mL del vino tinto previamente descrito en el apartado 4.1.1 (dosis equivalente a ~ 405 mg de polifenoles), en 3 tomas de 75 mL cada 8 h. Tras ello, el sistema se mantuvo con medio nutritivo durante seis días. Al cabo de este tiempo, se realizó una segunda simulación de ingesta de vino, en este caso con el sistema inoculado con las heces del voluntario V2. Para evaluar el efecto del etanol en la composición y actividad metabólica de la microbiota intestinal, se realizó una nueva simulación en las mismas condiciones pero alimentando el sistema con un vino sintético.

Durante todo el estudio, se recogieron muestras de los tres compartimentos del colon, en distinto tiempos: período de estabilización (SP), inmediatamente antes de la ingesta de vino (día -1), tras 8 h del inicio de la ingesta de vino (día 0), tras completar la ingesta completa de vino (día 1), y 24 h después del comienzo del lavado con medio nutritivo (día 2). Se tomó un alícuota de las muestras para su siembra en placa y el posterior recuento de poblaciones bacterianas. Otro alícuota de las muestras se centrifugó a 14000 rpm durante 10 min a 4 °C y ambos, pellet y sobrenadante, se almacenaron a -20 °C para su posterior análisis mediante RT-PCR y análisis químico (metabolitos fenólicos, ácidos grasos de cadena corta (AGCC) y amonio), respectivamente.

4.4.2.1. Análisis dirigido de compuestos fenólicos por UPLC-ESI-TQ MS/MS

El análisis dirigido de metabolitos fenólicos se llevó a cabo mediante UPLC-ESI-TQ MS/MS. Los sobrenadantes obtenidos a partir de la centrifugación de los alícuotas de cada muestra se filtraron utilizando un filtro de 0.22 µm, y se añadió un patrón interno (ácido 4-hidroxibenzoico 2,3,5,6-d4) a una concentración final de 2,5 ppm. Para la cuantificación de los metabolitos fenólicos se utilizó el método de Jimenez-Girón y col. (2013), previamente descrito en el apartado 4.2.1.2.

4.4.2.2. Análisis de las poblaciones bacterianas mediante recuento de colonias en medios selectivos

Los alícuotas de las muestras recogidos en los distintos compartimentos del SIMGI se diluyeron de forma seriada (10^{-1}) en solución fisiológica, y se sembraron en cuatro tipos de medios selectivos:

Wilkins-Chalgren agar (BD, Franklin Lakes, NJ, EE.UU.) para anaerobios totales, TSA (BD, Franklin Lakes, NJ, EE.UU.) para aerobios totales, MRS (Pronadisa, Madrid, España), para las bacterias lácticas y MacConkey agar (BD, Franklin Lakes, NJ, EE.UU.) para Enterobacterias. Las placas se incubaron a 37 °C durante 48 h en anaerobiois (Bactron anaeróbico / Cámara Ambiental, SHELLAB, Cornelius, OR, EE.UU.), a excepción de las placas de TSA que se incubaron en condiciones aerobias.

4.4.2.3. Análisis de las poblaciones bacterianas mediante q-PCR

La extracción de ADN de los "pellets" obtenidos a partir de las muestras recogidas de los distintos compartimentos del SIMGI (14000 rpm, 10 min, 4 °C), así como de aquellos procedentes de los cultivos puros de bacterias estándar (*Escherichia coli* ATCC 25922 y *Bacteroides fragilis* DSM 2151) (14000 rpm, 10 min, 4 °C), se llevó a cabo utilizando el kit de extracción QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Alemania), siguiendo el protocolo recomendado por el fabricante. Los "pellets (aproximadamente 100-150 mg) se lisaron químicamente con la solución tampón ASL suministrada por el fabricante del kit. Posteriormente, se eliminaron los posibles inhibidores de la PCR mediante el uso de la matriz polimérica InhibitEX, también proporcionada en el kit. Posteriormente la muestra se incubó con proteinasa k (QIAGEN, Hilden, Alemania) en un medio tamponado (tampón AL) y el lisado se eluyó por una columna de extracción "spin-column" (QIAGEN, Hilden, Alemania), que fija selectivamente el ADN. Después de consecutivos lavados de la columna con los tampones AW1 y AW2, suministrados en el kit, se eluyó el ADN de la columna con agua libre de nucleasas. Se confirmó la concentración y calidad del ADN determinando las relaciones Abs 260 nm/280nm y Abs 260 nm/230 nm, empleando un espectrofotómetro Nanodrop™ 1000 (Thermo Scientific, DE, EE.UU.). Las muestras procedentes de los cultivos puros de bacterias estándar se diluyeron de forma seriada y las diluciones se sembraron en placas de TSA (*E.coli*) y Modified Chopped Medium Agar (*B. fragilis*), con el fin de hacer un recuento de colonias y calcular el valor de UFC/mL.

Se utilizaron los cebadores que se muestran en la **tabla 11.** Las curvas de calibrado se realizaron a partir de los datos obtenidos de la cuantificación de diluciones seriadas a partir del ADN extraído de los cultivos puros de las bacterias de colección (*Escherichia coli* ATCC 25922 para los grupos *E.coli* y "All bacteria"; y *Bacteroides fragilis* DSM 2151 para el grupo *Bacteroides*. Las curvas de calibrado se

generaron mediante la representación gráfica de los valores de CT (Threshold cycles), en el eje de abscisas, frente a la concentración expresada en UFC/mL, en el eje de ordenadas.

Tabla 11. Cebadores utilizados para el análisis mediante RT-PCR

Grupo diana	Secuencia de los primers (5'-3')	Referencia
All bacteria	CGG TGA ATA CGT TCC CGG TAC GGC TAC CTT GTT ACG ACT T	Furet y col. (2009)
Bacteroides	GAG AGG AAG GTC CCC CAC CGC TAC TTG GCT GGT TCA G	Guo y col. (2008)
E. coli	CAT GCC GCG TGT ATG AAG AA CGG GTA ACG TCA ATG AGC AAA	Huijsdens y col. (2002)

La amplificación y detección de ADN de origen bacteriano en las muestras procedentes de los diferentes compartimentos del SIMGI, se llevó a cabo utilizando un equipo de PCR a tiempo real *ViiA™ 7* de Applied Biosystems® (Life Technologies, TX, USA) (**Figura 22**). El volumen final de cada reacción de amplificación fue de 10 µL incluyendo 5 µL de SYBER® Select Master Mix (Life Technologies, TX, USA), 200µL de cada cebador (10 µM), 600 µL de agua purificada libre de nucleasas (Sigma Aldrich, St. Louis, MO, EE.UU.) y 4µL de DNA. El programa de amplificación fue el siguiente: un primer ciclo a 50 °C durante 2 min, un ciclo a 95 °C durante 10 min, y 40 ciclos a 95 °C durante 30 s y 60 °C durante 1 min. Finalmente, se programó una curva de "melting" para comprobar la especificidad de los cebadores (95 °C durante 15 s y 60 °C durante 1 min, seguido de una rampa de temperatura de 0,05 °C/min, manteniendo finalmente una temperatura de 95 °C durante 15 s).

Figura 22. Equipo de PCR cuantitativa a tiempo real ViiA™ 7 empleado.

4.4.2.4. Análisis de amonio en los productos de digestión

La concentración de amonio se determinó utilizando un medidor de pH/ion selectivo (modelo 7320 (WTW, Weilheim, Alemania) acoplado a un electrodo selectivo de iones de amonio (WTW, Weilheim, Alemania). En primer lugar, el equipo se calibró utilizando una solución patrón de amonio (10 g/L), a las concentraciones de 0,1, 1, 10 y 100 mg de NH_4^+/ L. Para su evaluación en este medidor, la muestra (0,5 mL) se diluyó con agua desionizada (12 mL) y se mezcló con un 2% de la solución de acondicionado. La mezcla se midió inmediatamente a 25 °C y el resultado obtenido se expresó como NH_4^+/ L.

4.4.2.5. Análisis de ácidos grasos de cadena corta (AGCC) en los productos de digestión

Los AGCC se determinaron mediante micro-extracción en fase sólida (SPME) acoplada a cromatografía de gases y espectrometría de masas (GCMS), utilizando el método desarrollado por Bianchi y col. (2011), con algunas modificaciones. Brevemente, se mezclaron 290 µl del sobrenadante obtenido de cada muestra precedente de los distintos compartimentos del SIMGI, 10 µl de una solución de patrón interno ácido 2-metilvalérico (10 g/L), y 30 µl de una solución de H_2SO_4 0,9 M (pH=2). 100 µl de la mezcla se transfirieron a un vial de 20 mL cerrado herméticamente, y se procedió a la micro-extracción automática mediante un sistema CombiPAL (CTC Analytics AG, Zwingen, Suiza) con una fibra de SPME DVB/CAR/PDMS de 50/30 µm y 2 cm de longitud (Supelco, Bellefonte, PA). La extracción se llevó a cabo a 40 °C y durante 25 min. La desorción se llevó a cabo en el inyector del sistema de GC-MS utilizado (Agilent 7890A y Agilent MS 5975C, Palo Alto, CA, EE.UU.) durante 2 min a 260 °C en modo “Splitless”.

La separación cromatográfica se llevó a cabo en una columna capilar DB-FFAP (30 m x 0,25 mm x 0,25 m) de Agilent Technologies (Palo Alto, CA, EE.UU.). Se utilizó helio como gas portador a una velocidad de flujo de 1 mL/min. La temperatura del horno se mantuvo inicialmente a 100 °C durante 5 min, y después se aumentó a razón de 5 °C por min hasta 250 °C, temperatura que se mantuvo durante 12 min. Las adquisiciones se realizaron en modo SCAN (35-350 uma) y en modo de impacto electrónico (70 eV). Las temperaturas de la línea de transmisión, cuadrupolo y la fuente de ionización fueron 270

°C, 150 °C y 230 °C, respectivamente. La identificación de compuestos se llevó a cabo mediante la comparación con los tiempos de retención y espectros de masas de los compuestos de referencia de la biblioteca de espectros de masas NIST 2.0. Los datos cuantitativos se obtuvieron mediante el cálculo del área del pico de cada compuesto, en relación al del estándar interno (ácido 2-metilvalérico). Las curvas de calibrado de cada compuesto (ácido acético, ácido propiónico, ácido 2-metilpropiónico, ácido butanoico, ácido 2-metilbutanoico, ácido pentanoico, ácido hexanoico, ácido octanoico y ácido decanoico) se obtuvieron mediante el análisis de diluciones seriaddas de una solución estándar con todos los AGCC (5000 mg/L).

4.5. Análisis estadístico

Para el tratamiento estadístico de los resultados se utilizaron los siguientes métodos estadísticos:

- El Análisis de Componentes Principales (PCA) para examinar la relación entre las variables analizadas y diferenciar grupos de datos en función de dichas variables.
- El test paramétrico de la t-Student para datos relacionados y el correspondiente test no paramétrico de WILCOXON, para evaluar las diferenciar entre los resultados antes y después de los distintos tratamientos aplicados.
- El test de SHAPIRO-WILK para comprobar la distribución normal del conjunto de datos analizado.
- El Análisis de la Varianza (ANOVA) y el posterior test de la Mínima Diferencia Significativa (LSD) para comparar los valores medios de las variables y poder evaluar el efecto de distintos factores sobre éstas.

Para el procesamiento estadístico de los datos se utilizaron los programas: STATISTICA para Windows, versión 7.1 (StatSoft, Inc., 2005, www.statsoft.com) y el Microsoft Office EXCEL para Windows, versión 2010 (Microsoft, 2010, http://office.microsoft.com).

RESULTADOS

5. RESULTADOS

En esta sección se exponen los resultados obtenidos durante la Tesis Doctoral en base a la hipótesis y a los objetivos propuestos. Estos resultados se han recogido en 7 publicaciones científicas: 3 en revistas incluidas en el Science Citation Index (SCI), 3 enviadas para su publicación y 1 en fase de preparación.

Además, durante la realización de la presente Tesis Doctoral se ha realizado un artículo de revisión (Dueñas, M.; **Muñoz-González, I**.; Cueva, C.; Jiménez-Girón, A.; Sánchez-Patán, F.; Santos-Buelga, C.; Moreno-Arribas, M.V.; Bartolomé, B. 'Studies on modulation of gut microbiota by components of some dairy fermented foods: a focus on polyphenols', *BioMed Research International,* [en proceso de publicación], que recoge los principales avances sobre la modulación de la microbiota gastrointestinal por compuestos fenólicos presentes en el vino y otros alimentos fermentados.

Por último, los resultados derivados de esta Tesis Doctoral se han publicado en dos artículos científicos recogidos en revistas Open Access (Jiménez-Girón, A, **Muñoz-González, I**., Martín-Álvarez, P.J., Bartolomé, B., Moreno-Arribas, M. V**. '**Moderate intake of red wine promotes a significant increase of phenolic metabolites in human faeces'. *Nutrition and Ageing* 2014, *2*, 151-156; y Jiménez-Girón, A., **Muñoz-González, I.**; Martín-Álvarez, P.J.; Moreno-Arribas, M.V.; Bartolomé, B. 'Towards the Faecal Metabolome Derived from Moderate Red Wine Intake', *Metabolites*, 2014, special issue [en proceso de publicación].

5.1. Efecto del consumo de moderado de vino tinto sobre perfil metabólico fecal humano

Son numerosos los estudios epidemiológicos, de intervención e *in vitro* que evidencian efectos beneficiosos del consumo moderado de vino, especialmente de vino tinto, frente a enfermedades cardiovasculares. Sin embargo, su posible efecto sobre la salud digestiva apenas se ha abordado, y son escasos los estudios *in vivo*, en humanos, llevados a cabo con este fin.

El vino es considerado como uno de los alimentos con mayor contenido en polifenoles y es a estos compuestos, a los que se les atribuyen particularmente los efectos saludables derivados del consumo moderado del vino frente a distintas patologías. Sin embargo, una vez ingeridos, los polifenoles sufren un extenso metabolismo en el organismo humano, por lo que su potencial bioactivo parece deberse más a los metabolitos generados, especialmente por la acción de la microbiota intestinal, que a los compuestos presentes originariamente en el alimento (Monagas y col. 2010; Williamson y Clifford, 2010). Conocer los posibles efectos que los polifenoles del vino pueden ejercer a nivel intestinal pasa necesariamente por determinar el contenido de los diversos metabolitos fenólicos presentes en el lumen intestinal tras el consumo de vino, lo que permitirá avanzar también en el conocimiento de cómo la microbiota intestinal es capaz de metabolizar los componentes del vino. Por otro lado, dada la gran dificultad para disponer de muestras de contenido intestinal, el análisis del material fecal constituye una buena aproximación para el estudio de los efectos del consumo de un alimento (i.e., vino) (Laparra y Sanz, 2010) a nivel intestinal. Por tanto, dentro del objetivo general de profundizar en los efectos del consumo moderado de vino en la salud gastrointestinal, nuestro primer objetivo concreto fue el de evaluar los cambios en los metabolitos fenólicos y no fenólicos, y en otros parámetros relacionados (marcadores inmunológicos, microbiota intestinal) en heces humanas, tras el consumo moderado de vino en voluntarios sanos (Objetivo 1). En este apartado se recogen los resultados relativos al metaboloma fecal, y en los apartados siguientes, los relativos al estudio de los marcadores inmunológicos (Apartado 5.2) y microbiota intestinal (Apartado 5.3).

Dada la complejidad de las muestras fecales, el análisis del perfil metabólico fecal podría requerir la aplicación previa de técnicas de preparación de la muestra, como la extracción en fase sólida (SPE), con el objetivo de eliminar posibles interferencias y/o concentrar la muestra, reduciendo el efecto de la matriz e incrementando la sensibilidad del método de análisis posterior (Urpí-Sarda y col., 2009). Por

otro lado, entre las técnicas más avanzadas, la cromatografía de líquidos de ultra eficacia (i.e., UPLC, UHPLC) -que opera con columnas que contienen partículas de pequeño tamaño (< 2 µm)- acoplada a modernos detectores de masa (i.e., TQ MS/MS, TOF MS), es sin duda la técnica más adecuada para un análisis rápido, sensible y robusto de metabolitos fenólicos (Sánchez-Patán y col., 2011). Pero, además de estudiar los cambios en los metabolitos fenólicos previamente identificados (análisis dirigido), es posible realizar un análisis no dirigido seleccionando las señales *m/z* que resultan estadísticamente significativas en la comparación entre muestras, y procediendo a su identificación posterior. Este es el fundamento de la aproximación metabolómica, que está resultando muy útil en la búsqueda de biomarcadores nutricionales de la ingesta de alimentos, mecanismos de acción, efectos fisiológicos, etc. (Llorach y col., 2010).

Por tanto, en este apartado se han abordado los siguientes sub-objetivos concretos:

1. Optimizar y evaluar la eficacia del empleo de una técnica de extracción en fase sólida (SPE) como paso previo al análisis por UPLC-ESI-TQ MS/MS (**Publicación 5.1.1**).

2. Evaluar el efecto del consumo moderado de vino sobre el perfil de metabolitos fenólicos en muestras fecales, procedentes de un estudio de intervención nutricional con vino tinto, mediante un análisis dirigido por UPLC-ESI-TQ MS/MS (**Publicación 5.1.2).**

3. Evaluar el efecto del consumo moderado de vino sobre el metaboloma fecal mediante el análisis no dirigido de muestras fecales, procedentes de un estudio de intervención nutricional con vino tinto, por UHPLC-TOF MS (**Publicación 5.1.3).**

A continuación, se presentan los resultados de estos trabajos en forma de publicaciones científicas.

<u>Publicación 5.1.1</u> Evaluación de la SPE como técnica preparativa de la muestra para el análisis de muestras fecales humanas

Evaluation of SPE as Preparative Technique for the Analysis of Phenolic Metabolites in Human Feces

Irene Muñoz-González, Fernando Sánchez-Patán, Ana Jiménez-Girón, Carolina Cueva, María Monagas, Pedro J. Martín Álvarez, M. Victoria Moreno-Arribas, Begoña Bartolomé. *Food Analytical Methods*, **2014**, 7: 844-853

Resumen:

En este trabajo, se ha evaluado una metodología de extracción en fase sólida (SPE) como fase previa al análisis de metabolitos fenólicos en muestras fecales mediante UPLC-DAD-ESI TQ MS. De los rellenosevaluados, el Oasis ® HLB permitió una mayor recuperación de los patrones fenólicos ensayados. Además, la acidificación de la muestra (0,4 M de HCl, concentración final) aumentó considerablemente los valores de recuperación obtenidos. La eficacia del proceso (concentración $_{con\ SPE}$ / concentración $_{sin\ SPE}$) calculada tras la aplicación de la SPE a una mezcla de patrones compuesta por ácido gálico, ácido protocatéquico, ácido cafeico, ácido benzoico, ácido 3–fenilpropiónico, (+)-catequina, (-)-epicatequina, procianidina B2, y ácido 2,3,5,6 d_4 4-hidroxibenzoico fue aceptable (> 90 %) para todos los compuestos, excepto para la procianidina B2 (26 %). La metodología de la SPE desarrollada, se aplicó a un lote de muestras de heces precedentes de participantes en un estudio de intervención nutricional con vino tinto. El análisis de las muestras permitió la identificación de 25 metabolitos fenólicos, incluyendo metabolitos intermedios (derivados de fenil-γ-valerolactona y ácido fenilvalérico), y metabolitos finales (fenoles simples, ácidos hidroxifenilpropiónico, hidroxifenilacético, hidroxicinámico e hidroxibenzoico). La mayoría de los compuestos identificados (n=14) mostraron valores de recuperación entre el 85 y el 115%. Aunque para algunos compuestos (n=4) las recuperaciones fueron mayores al 115 %, otros fueron parcialmente retenidos en los cartuchos [ácido 4-O- metilgálico, ácido siríngico, y ácido 4-hidroxi-5-(3',4'-dihidroxifenil-valérico)], con recuperaciones menores al 85 %, lo que limitaba la utilización de la SPE para este tipo de análisis, y en consecuencia nos hizo descartar, su utilización. Por último, este trabajo muestra los valores de concentración de metabolitos fenólicos en un conjunto aleatorio de muestras fecales de voluntarios sanos (n=15), no sometidas a SPE. El análisis de estas muestras resultó en una gran variabilidad interindividual, lo que podría ser atribuido a diferencias en la composición de la microbiota intestinal humana.

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Abstract Solid phase extraction (SPE) methodology has been evaluated as a cleanup strategy prior to the analysis of phenolic metabolites in fecal samples by UPLC–DAD–ESI–TQ MS. Among the sorbents tested, Oasis® HLB led to the higher phenolic standard recoveries. Sample acidification (0.4 M HCl, final concentration) before SPE considerably improved standard recoveries. Values of the process efficiency ($C_{SPE}/C_{Without\ SPE}$) for a standard solution containing gallic acid, protocatechuic acid, caffeic acid, benzoic acid, 3-phenylpropionic acid, (+)-catechin, (−)-epicatechin, procyanidin B2, and 4-hydroxybenzoic 2,3,5,6 d_4 acid were acceptable (>90 %) for all compounds, except for procyanidin B2 (26 %). The developed SPE methodology was applied to fecal samples of individuals subjected to a wine intervention study. Phenolic metabolites, including intermediate metabolites (phenyl-γ-valerolactones and phenylvaleric acid derivatives) and end products (simple phenols, hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, and hydroxybenzoic acids) were identified. Most of the compounds (n=14) exhibited values of process efficiency between 85 and 115 %. Although some compounds (n=4) showed process efficiency>115 %, there was a group of metabolites (4-*O*-methylgallic acid, syringic acid, and 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid) whose process efficiency was <85 %, which represented a serious limitation and made us to discard SPE as a preparative technique for the analysis of these phenolic metabolites. Finally, the paper reports the concentrations of phenolic metabolites in a randomized set of human fecal samples from healthy volunteers (n=15) without any previous SPE application. Large inter-individual variability was observed, which was attributed to differences in human gut microbiota composition.

Keywords Solid phase extraction · SPE · Phenolic metabolites · Human feces · UPLC

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Introduction

Phenolic compounds or polyphenols are a large group of secondary plant metabolites within which more than 500 are reported to occur in foodstuffs (Tarascou et al. 2010). Over the past decades, they have attracted a large interest because of their potential beneficial health effects, especially in the prevention of diseases associated with oxidative stress such as cancer, cardiovascular, inflammatory, and neurodegenerative diseases, as suggested by clinical trials and epidemiological studies (Scalbert et al. 2005; Hollman et al. 2011; Pasinetti 2012).

Current research indicates that the majority of dietary plant polyphenols (90–95 %) are not bioavailable, have scarce absorption in the small intestine, and reach the colon where they are widely metabolized by gut microbiota into phenolic acids and other metabolites that could be further absorbed (Selma et al. 2009; Williamson and Clifford 2010). For instance, for flavan-3-ols, catabolites include intermediate (i.e., phenyl-γ-valerolactones and phenylvaleric acid derivatives) and end products (simple phenols, hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, and hydroxybenzoic acids) (Touriño et al. 2011; Sánchez-Patán et al. 2012). Moreover, microbial-derived metabolites could be even more bioactive than their precursors (Monagas et al. 2010). The

health-promoting activities of phenolic metabolites and interactions between them and colonic microbiota are of current research interest (Tomás-Barberán and Andrés-Lacueva 2012). Therefore, determination of phenolic acids in feces is an interesting approach for the study of the phenolic catabolism by gut microbiota, allowing a better knowledge about the physiological action of polyphenols both at the gut level and systemically.

The most commonly used analytical methods for the determination of microbial phenolic metabolites in biological fluids employ gas chromatography–mass spectrometry (Jenner et al. 2005; Grün et al. 2008; Muñoz-González et al. 2012; Stalmach et al. 2013) and liquid chromatography (LC) coupled to diode-array (DAD) or electrochemical detectors and particularly to tandem mass spectrometry (ESI–MS/MS) (Gonthier et al. 2006; Appeldoorn et al. 2009; Urpi-Sarda et al. 2009). The efficiency of separation and the sensitivity of MS detection by LC–MS/MS methodologies can be largely improved by the use of ultraperformance liquid chromatography (UPLC), which operates with smaller particle size (<2 μm) sorbent materials and internal diameter columns (1–2.1 mm) than LC at very high pressures (up to 15,000 psi) (Nagy et al. 2009; Novakova et al. 2010). The UPLC technique coupled to MS detection is finding good application in the analysis of phenolic compounds in biological samples (Serra et al. 2009; Sánchez-Patán et al. 2011).

Despite the great inter-individual variability, the main difficulty of the analysis of phenolic metabolites in feces resides in the matrix complexity, which creates interference in the detection and accurate quantification by MS. Because of this, it is common to apply sample preparation techniques to reduce matrix effects and retain interfering compounds such salts, proteins, cells, lipids, or lipoproteins previous to LC–MS analysis in order to obtain repeatable and reliable results (Kole et al. 2011; Chambers et al. 2007). Among others, solid phase extraction (SPE) could be a good choice as a technique for sample cleanup and matrix effect reduction in fecal samples, as it has been successfully applied in other biological fluids such as urine and blood (Urpi-Sarda et al. 2009; Tulipani et al. 2013).

The aim of this paper was to test the SPE as a preparative sample technique for the analysis of phenolic metabolites in human feces. After selection of the sorbent and certainty of the need of sample acidification, the developed SPE methodology was applied to some fecal samples from a human wine intervention study. Although MS response of some compounds seemed to be improved after SPE, the process efficiency (percent) was markedly low for some intermediate metabolites, which could limit the applicability of this sample preparative technique. Finally, the paper reports the results of a randomized study (n=15) of basal levels on phenolic metabolites in human feces in which samples were not subjected to any preparative technique before UPLC–DAD–ESI–TQ MS analysis. Results emphasized the diversity and inter-individual variability of phenolic metabolites in this physiological material.

Experimental

Phenolic Standards

Phenolic acid standards used in the study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), Phytolab (Vestenbergsgreuth, Germany), or Extrasynthese (Genay, France). LC grade solvents were purchased from Lab-Scan (Gliwice, Poland).

A standard solution contained phenolic acids (gallic acid, 125 μg mL^{-1}; protocatechuic acid, 100 μg mL^{-1}; caffeic acid, 125 μg mL^{-1}; benzoic acid, 250 μg mL^{-1}, and 3-phenylpropionic acid, 500 μg mL^{-1}) as well as flavan-3-ols ((+)-catechin, 100 μg mL^{-1}; (−)-epicatechin, 100 μg mL^{-1}; and procyanidin B2, 250 μg mL^{-1}) and a deuterated MS standard (4-hydroxybenzoic 2,3,5,6 d_4 acid, 250 μg mL^{-1}) in acetonitrile-water (1:4, *v/v*) was prepared.

Fecal Samples

Human fecal samples (n=15) from healthy volunteers with normal dietary habits were collected at the Ramón y Cajal (RyC) Hospital (Madrid, Spain) after confirming that they were free of parasites or pathogenic bacteria. Additionally, a fecal sample from a human intervention study, consisting of a 2-week low polyphenols diet followed by 1 month moderate red wine consumption (250 mL/day), was used in this study.

Samples were stored at −20 °C until they were used. For preparation of the fecal solutions, feces were thawed at room temperature and 4 g were mixed with 40 mL of sterile saline solution 0.9 %. The mixture was vortexed until homogenization and centrifuged twice (10 min, 10,000 rpm, 4 °C) until the supernatants (fecal solutions) were visually clear. Fecal solutions were then filtered with a filtration unit Stericup® Express™ PLUS 0.22 mm, Millipore (Billerica, MA, USA).

SPE

Selection of SPE Cartridge

The following cartridges were tested: Oasis® HLB 60 mg 3 cc (Waters, Milford, MA, USA) composed by a hydrophilic–lipophilic-balanced reversed-phase sorbent with a divinylbenzene vinylpyrrolidone structure and an *N*-vinylpyrrolidone group, Oasis® MCX 60 mg 3 cc (Waters, Milford, MA, USA) composed by a mixed-mode cation exchange sorbent with a divinylbenzene vinylpyrrolidone structure with sulfonic groups, Oasis® MAX 60 mg 3 cc (Waters, Milford, MA, USA) composed by a mixed-mode polymeric sorbent with anion-exchange groups, and Bond Elut Plexa™ 60 mg 3 cc (Agilent Technologies, Waldbronn, Germany) composed by a nonpolar divinylbenzene-based neutral polymeric sorbent.

SPE experiments were performed using an SPE vacuum manifold system with a 12 positions rack. The generic protocols of extraction recommended by the cartridges manufacturers were performed. Prior to extraction, sorbents were conditioned with 2 mL MeOH and equilibrated with 2 mL of MilliQ water for HLB, MAX, and PLEXA and with 2 mL of a 2 % HCOOH solution in MilliQ water for MCX. Two milliliter of MilliQ water+100 μL of the standard solution were loaded on each type of sorbent (MCX, MAX, HLB, and PLEXA). Washing was performed with 2 mL of 2 % HCOOH for MCX, HLB, and PLEXA and of 5 % NH_4OH solution in MilliQ water for MAX. The elution step was assessed with 2 mL of MeOH for HLB, MCX, and PLEXA and with 2 mL of 2 % HCOOH solution in MeOH for MAX. Eluates from the elution step were filtered through a 0.22 μm pore size filter and diluted (1:1, *v/v*) with MilliQ water in order to keep an accurate aqueous:organic proportion (similar to the initial mobile phase composition) for further analysis by UPLC–DAD–ESI–TQ MS. SPE experiments were carried out in triplicate.

For calculating the recovery, a mixture of 2 mL of MilliQ water+100 μL of the standard solution was analyzed by UPLC–DAD–ESI–TQ MS ("without SPE" sample).

Experiment of Sample Acidification Prior to SPE

Two milliliter of MilliQ water+100 μL of the standard solution, with and without previous acidification (+68 μL of HCl 37 %, 0.4 M, final concentration), were loaded onto Oasis® HLB cartridges previously conditioning and equilibration as described above. The elution was then performed as indicated above, and the 2 ml of eluate was diluted (1:1, *v/v*) with MilliQ water before analysis by UPLC–DAD–ESI–TQ MS.

Application of SPE to Fecal Solution Spiked with Standard Compounds

A volume of 2 mL of an acidified (0.4 M HCl) fecal solution was spiked with 100 μL of the standard solution and loaded onto the Oasis® HLB cartridge for SPE extraction as indicated above (*pre-extraction spiked sample*). Parallel, 2 mL of the same acidified fecal solution were subjected to SPE as indicated above, and a volume of 100 μL of the standard solution was added to the SPE eluate (*post-extraction spiked sample*). Both eluates obtained were diluted (1:1, *v/v*) with MilliQ water before UPLC–DAD–ESI–TQ MS analysis.

Application of SPE to Different Load Volumes of Fecal Solution

Preconcentration capacity of the SPE methodology was evaluated by loading two different volumes (2 and 6 mL) of an acidified (0.4 M HCl) fecal solution onto Oasis® HLB for SPE extraction as indicated above. The eluates (2 mL) were subjected to the same dilution (1:1, *v/v*) with MilliQ water before analysis by UPLC–DAD–ESI–TQ MS.

UPLC–DAD–ESI–TQ MS Analysis of Phenolic Metabolites

A UPLC system coupled to an Acquity PDA eλ photodiode array detector (DAD) and an Acquity TQD tandem quadrupole mass spectrometer equipped with Z-spray electrospray interface (UPLC–DAD–ESI–TQ MS) (Waters, Milford, MA) was used. Separation was performed on a 2.1×100 mm id., 1.7 μm, BEH C18 column (Waters, Milford, MA) at 40 °C. The mobile phases were 2 % acetic acid in water (A) and 2 % acetic acid in acetonitrile (B). A gradient program was applied at a flow rate of 0.5 mL/min as follows: 0 min, 0.1 % B; 1.5 min, 0.1 % B; 11.17 min, 16.3 % B; 11.5 min, 18.4 % B; 14 min, 18.4 % B; 14.1 min, 99.9 % B; 15.5 min, 99.9 % B; 15.6 min, 0.1 % B; and 18 min, 0.1 % B (Sánchez-Patán et al. 2011). The injection volume was 2 μL. DAD detector was operated in the 250–420 nm wavelength range at a 20 point/s rate and 1.2 nm resolution. The ESI parameters were set as follows: capillary voltage, 3 kV; source temperature, 130 °C; desolvation temperature, 400 °C; desolvation gas (N2) flow rate, 750 L/h; cone gas (N2) flow rate, 60 L/h. The ESI was operated in negative ionization mode. For identification and quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound and using external calibration curves. MRM transitions targeted (corresponding to flavan-3-ols, phenolic acids, and other metabolites) were optimized previously (Sánchez-Patán et al. 2011; Jiménez-Girón et al. 2013): (+)-catechin (289>245), (−)-epicatechin (289>245), procyanidin B2 (577>289), 3,4-dihydroxymandelic acid (183>137), 4-hydroxymandelic acid (167>123), 4-hydroxy-3-methoxymandelic acid (197>137), 3-hydroxymandelic acid (167>121), 3-hydroxy-4-methoxymandelic acid (197>137), mandelic acid (151>107), gallic acid (169>125), 3,5-dihydroxybenzoic acid (153>109), protocatechuic acid (153>109), 3-*O*-methylgallic acid (183>168), 4-hydroxybenzoic acid (137>93), 4-*O*-methylgallic acid (183>168), 3-hydroxybenzoic acid (137>93), vanillic acid (167>152), syringic acid (197>182), 3,4-dimethoxybenzoic acid (181>107), benzoic acid (121>77), salicylic acid (137>93), 4-methoxy benzoic acid (151>107), 3,4,5-trimethoxybenzoic acid (211>167), 3-methoxybenzoic acid (151>107), phloroglucinol (125>83), pyrogallol (125>79), catechol/pyrocatechol (109>81), 4-methyl catechol (123>108), 4-ethylcatechol (137>122), tyrosol (137>106), 4-hydroxyhippuric acid (194>100), hippuric acid (178>134), 3,4-dihydroxyphenylacetic acid (167>123), 4-hydroxy-phenylacetic acid (151>107), 3-hydroxyphenylacetic acid (151>107), 4-hydroxy-3-methoxyphenylacetic acid

(181>137), phenylacetic acid (135>91), 3,4-dimethoxyphenyl acetic acid (195>136), 4-methoxyphenylacetic acid (165>106), 3-(3,4-dihydroxyphenyl)-propionic acid (181>137), 3-(4-hydroxyphenyl)-propionic acid (165>121), 3-(3-hydroxyphenyl)-propionic acid (165>121), 3-(3,4-dimethoxyphenyl)-propionic acid (209>150), phenylpropionic acid (149>105), 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid (225>163), 4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid (209>147), 4-hydroxy-5-(phenyl)-valeric acid (193>175), 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (207>163), 5-(3′-hydroxyphenyl)-γ-valerolactone (191>147), 5-(4′-hydroxyphenyl)-γ-valerolactone (191>147), caffeic acid (179>135), *p*-coumaric acid (163>119), ferulic acid (193>134), *m*-coumaric acid (163>119), isoferulic acid (193>134), *trans*-cinnamic acid (147>103), 3,4,5-trimethoxycinnamic acid (237>103), and 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol (291>247). As indicated in previous studies (Sánchez-Patán et al. 2011; Jiménez-Girón et al. 2013), phenolic metabolites were quantified using calibration curves of the proper standards or of other chemically related compounds when the standards were not commercially available. Data acquisition and processing was realized with MassLynx 4.1 software.

Accuracy (percent of nondeviation) of the present methodology ranged between 80 and 126 % and met acceptance criteria (<20 %) for most of the phenolic compounds (Sánchez-Patán et al. 2011), and the precision (percent RSD) was lower than 15 % ranging from 0.01 to 13.4 % (Sánchez-Patán et al. 2011).

Results and Discussion

Sorbent Selection and Sample Conditioning

Preliminary experiments were performed in order to select the most favorable cartridge and sample pretreatment. Four SPE cartridges with different sorbent characteristics (Oasis®HLB, Oasis®MCX, Oasis®MAX, and Bond Elut Plexa™) were tested. The Oasis®HLB cartridge showed the best ability to recuperate the analytes from a standard solution containing phenolic acids (gallic acid, protocatechuic acid, caffeic acid, benzoic acid, and phenylpropionic acid), flavan-3-ols ((+)-catechin, (−)-epicatechin, and procyanidin B2), and a deuterated standard (4-hydroxybenzoic 2,3,5,6 d_4 acid). For example, the recovery of 4-hydroxybenzoic 2,3,5,6 d4 was 58, 51, 33, and 50 % with the cartridges Oasis®HLB, Oasis®MCX, Oasis®MAX, and Bond Elut Plexa™, respectively. Similarly, other authors found the highest recoveries and lowest relative standard deviations for Oasis®HLB cartridges in the application of SPE for analysis of isoflavones to urine and plasma samples (Baranowska et al. 2011).

Once the Oasis HLB cartridge was selected, the same standard solution was extracted at acidic (0.4 M HCl) or neutral conditions in order to evaluate the best retention conditions for the loading step. Acidification of samples prior to SPE considerably enhanced the recovery of the majority of the phenolic compounds. For example, the recovery of 4-hydroxybenzoic 2,3,5,6 d_4 acid was from 58 to 96 % after sample acidification. Table 1 reports the values of process efficiency or recovery ($C_{SPE}/C_{Without\ SPE}$) for the acidified standard solution submitted to SPE extraction on Oasis®HLB cartridges. Values of process efficiency were acceptable (>90 %) for all compounds, except for procyanidin B2 (26 %), which could be due to its low polar structure and to interactions between the molecule and the reversed-phase sorbent of the HLB cartridge. Other authors have also obtained good recoveries in the application of Oasis®HLB cartridges for the analysis of wine phenolic compounds (Pérez-Magariño et al. 2008; Silva 2011), some of which are also included in our study.

Effect of Fecal Matrix

For the evaluation of the effect of the fecal matrix, a fecal solution was spiked with the standard solution before (pre-extraction spiked sample) and after (post-extraction spiked sample) SPE extraction on Oasis®HLB cartridges. Table 2 reports the metabolite concentrations of these two samples, together with the calculations of process efficiency and fecal matrix effect on UPLC–MS and on SPE (Matuszewski et al. 2003). Data of the standard solution without being subjected to SPE (*spiking solution*) (Table 2) corresponded to that previously reported in Table 1.

The fecal matrix seemed not to compromise the SPE efficiency ($C_{Spiked\ sample}/C_{Spiking\ solution}$) for the phenolic acids tested, as their values were >90 % (Table 2). In relation to 3-

Table 1 Concentration values (mean±standard deviation) of the different phenolic compounds of the acidified standard solution with and without the application of SPE

Compound	Without SPE (μg mL^{-1})	SPE (μg mL^{-1})	Process efficiency (%)[a]
Gallic acid	3.27±0.08	2.93±0.21	90
Protocatechuic acid	2.91±0.05	2.95±0.03	101
Caffeic acid	0.995±0.072	0.944±0.021	95
Benzoic acid	8.96±0.23	9.01±0.11	100
3-Phenylpropionic acid	13.8±0.6	13.8±0.1	100
(+)-Catechin	2.56±0.15	2.40±0.01	94
(−)-Epicatechin	2.19±0.11	2.01±0.08	92
Procyanidin B2	2.91±0.10	0.767±0.108	26
4-Hydroxybenzoic 2, 3, 5, 6 d_4 acid	8.54±0.43	8.17±0.27	96

[a] Calculated as: $(C_{SPE}/C_{Without\ SPE})\times 100$

phenylpropionic acid, further analysis indicated that this compound was found in the fecal solution per se, which explained that its corresponding value for process efficiency were >>100 %. Only (+)-catechin (80 %), (−)-epicatechin (70 %), and especially procyanidin B2 (8 %) showed values of process efficiency<90 %, indicating the occurrence of interactions between flavan-3-ol and the Oasis®HLB sorbent, favoring the retention of these compounds in the cartridge. Therefore, this SPE procedure appeared not to be accurate for the analysis of flavan-3-ol compounds in fecal solutions.

The ratio between concentrations corresponding to the post-extraction spiked sample and to the fecal solution spiked with the standard solution ($C_{\text{Post-extraction spiked sample}}/C_{\text{Spiking solution}}$) was indicative of matrix effects on MS detection (Table 2). Except for 3-phenylpropionic acid, all values ranged between 97 and 106 %, indicating that the fecal matrix produced no effect in the detection and in the further quantification of these compounds by UPLC–DAD–ESI–TQ MS, at least when a twofold sample dilution was considered.

The ratio between concentrations corresponding to the spiked sample and the post-extraction spiked sample ($C_{\text{Spiked sample}}/C_{\text{Post-extraction spiked sample}}$) was indicative of matrix effect on SPE (Table 2). All values were >90 %, except for (+)-catechin, (−)-epicatechin, and especially procyanidin B2, as seen before for process efficiency values. Therefore, the SPE methodology developed only seemed adequate for the analysis of phenolic acids in fecal solution, at least for those included in the standard solution (gallic acid, protocatechuic acid, caffeic acid, benzoic acid, and phenylpropionic acid).

Application of SPE to the Analysis of Fecal Samples

Once developed and validated for a standard solution, the SPE methodology was applied to fecal samples that were expected to be rich in phenolic metabolites. They originated from a human intervention study that consisted on a daily intake of a red wine (250 mL) during four weeks (Muñoz-González, unpublished results). Table 3 shows the concentration values of phenolic compounds identified in one of these phenolic-rich fecal samples, without and after SPE. Two different volumes (2 mL and 3×2 mL) were applied to the Oasis®HLB cartridges in order to test the optimum volume of sample to be loaded on the cartridges to improve metabolite detection.

A total of 22 phenolic metabolites, including intermediate metabolites derived from the catabolism of flavan-3-ols (i.e., phenyl-γ-valerolactones and phenylvaleric acid derivatives) and end products (simple phenols, hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, and hydroxybenzoic acids) were identified in the fecal sample not subjected to SPE. On the other hand, a slightly larger number of compounds (23 metabolites for a 2 mL loaded volume and 25 metabolites for 3×2 mL volume) were identified in the eluates from SPE when the volume of loaded sample was increased. Among all metabolites identified, 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 5-(3′,4′,-dihydroxyphenyl)-γ-valerolactone, 3-(4-hydroxyphenyl)-propionic acid, 3-(3-hydroxyphenyl)-propionic acid, phenylacetic acid, phenylpropionic acid, and 4-hydroxy-5-(phenyl)-valeric acid were the most abundant. It is noteworthy that the occurrence of two valerolactones (5-(3′,4′,-dihydroxyphenyl)-γ-valerolactone and 5-hydroxyphenyl-γ-valerolactone) and

Table 2 Concentration values (mean±standard deviation) of the different phenolic compounds in pre-extraction and post-extraction spiked fecal samples

Compound	Spiking solution (μg mL^{-1})	Without SPE Pre-extraction spiked sample (μg mL^{-1})	SPE Post-extraction spiked sample (μg mL^{-1})	Process efficiency (%)[a]	Matrix effect on UPLC–MS (%)[b]	Matrix effect on SPE (%)[c]
Gallic acid	3.27±0.08	3.01±0.14	3.42±0.05	92	105	88
Protocatechuic acid	2.91±0.05	3.03±0.20	3.09±0.04	104	106	98
Caffeic acid	0.995±0.072	1.00±0.06	1.04±0.01	101	104	96
Benzoic acid	8.96±0.23	9.51±0.43	9.44±0.08	106	105	101
3-Phenylpropionic acid	13.8±0.6	18.6±0.9	19.6±0.7	135	142	95
(+)-Catechin	2.56±0.15	2.06±0.09	2.67±0.03	80	104	77
(−)-Epicatechin	2.19±0.11	1.54±0.09	2.13±0.05	70	97	72
Procyanidin B2	2.91±0.10	0.250±0.035	2.93±0.04	8	101	8
4-Hydroxybenzoic 2, 3, 5, 6 d_4 acid	8.54±0.43	8.16±0.46	8.28±0.14	96	97	98

[a] Calculated as: $(C_{\text{Pre-extraction spiked sample}}/C_{\text{Spiking solution}})\times 100$

[b] Calculated as: $(C_{\text{Post-extraction spiked sample}}/C_{\text{Spiking solution}})\times 100$

[c] Calculated as: $(C_{\text{Pre-extraction spiked sample}}/C_{\text{Post-extraction spiked sample}})\times 100$

Table 3 Concentration values (mean±standard deviation) of phenolic metabolites in a rich metabolite fecal solution with and without the application of SPE

Compound	Without SPE	SPE		SPE	
	Concentration (μg mL^{-1})	2 mL load		3×2 mL load	
		Concentration (μg mL^{-1})	Process efficiency (%)[a]	Concentration (μg mL^{-1})	Process efficiency (%)[a]
Gallic acid	tc	tc	–	tc	–
3,5-Dihydroxybenzoic acid	–	0.0229±0.0033	–	0.0216±0.0001	–
Protocatechuic acid	0.0401	0.0343±0.0009	85	0.0356±0.0021	89
3,4-Dihydroxyphenylacetic acid	0.977	1.09±0.05	112	0.978±0.138	100
3-*O*-methylgallic acid	0.0548	0.0546±0.0078	100	0.0541±0.0042	99
4-Hydroxybenzoic acid	0.130	0.116±0.001	89	0.126±0.004	97
4-*O*-methylgallic acid	0.00659	0.00423±0.00001	64	0.00339±0.00011	51
Catechol/pyrocatechol	0.0816	0.0870±0.0030	106	0.0762±0.0040	93
4-Hydroxyphenylacetic acid	0.457	0.409±0.058	90	0.432±0.061	90
3-(3,4-Hydroxyphenyl)propionic acid	–	–	–	0.0444±0.0062	–
Vanillic acid	0.0808	0.113±0.019	139	0.0970±0.0013	120
3-Hydroxyphenylacetic acid	2.07	1.83±0.11	89	1.82±0.02	88
Syringic acid	0.328	0.237±0.009	72	0.212±0.002	65
4-Hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid	0.882	0.0613±0.0100	7	0.0604±0.0046	7
5-(3′,4′,-Dihydroxyphenyl)-γ-valerolactone	1.84	3.50±0.09	190	3.08±0.08	167
p-Coumaric acid	0.0354	0.0325±0.0047	92	0.0339±0.0047	96
3-(4-Hydroxyphenyl)-propionic acid	1.50	1.43±0.13	96	0.990±0.099	66
3-(3-Hydroxyphenyl)propionic acid	17.7	15.9±0.1	90	12.4±0.1	70
Benzoic acid	0.286	0.244±0.034	85	0.299±0.008	104
Ferulic acid	tc	tc	–	0.0351±0.0006	–
Phenylacetic acid	1.35	1.30±0.17	97	1.14±0.01	85
5-Hydroxyphenyl-γ-valerolactone	0.0851	0.188±0.011	220	0.168±0.005	197
3-Phenylpropionic acid	6.40	5.54±0.03	87	4.56±0.32	71
4-Hydroxy-5-(phenyl)-valeric acid	8.13	7.54±0.05	93	7.43±0.39	91
Dihydroxyphenylpropan-2-ol	–	–	–	0.0140±0.0024	–

tc traces

[a] Calculated as: $(C_{SPE}/C_{Without\ SPE})\times 100$

two valeric acids (4-hydroxy-5-(phenyl)-valeric acid and 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid) that have been previously reported in batch culture fecal fermentations (Sánchez-Patán et al. 2012; Appeldoorn et al. 2009) but have not been reported in human fecal samples before. In addition, precursors such procyanidins and monomers (+)-catechin and (−)-epicatechin were not detected in these fecal samples previous to SPE, indicating that the microbial metabolism is complete after wine ingestion.

Values of SPE process efficiency (percent) (Table 3) were calculated for all identified compounds. In general, similar efficiency values were obtained for 2 mL and 3×2 mL loading volumes. Most compounds exhibited a process efficiency between 85 and 115 % (protocatechuic acid, 3,4-dihydroxyphenylacetic acid, 3-*O*-methylgallic acid, 4-hydroxybenzoic acid, catechol/pyrocatechol, 4-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, *p*-coumaric acid, 3-(4-hydroxyphenyl)-propionic acid, 3-(3-hydroxyphenyl)-propionic acid, benzoic acid, phenylacetic acid, phenylpropionic acid, and 4-hydroxy-5-(phenyl)-valeric acid) which indicated that their recovery from the SPE cartridges was almost complete. Some of these compounds were also present in the standard solution used in previous experiments; thus, the results from both standard solution and fecal samples were totally consistent. Other compounds showed process efficiency >115 % (3,5-dihydroxybenzoic acid, vanillic, 5-(3′,4′,-dihydroxyphenyl)-γ-valerolactone, and 5-hydroxyphenyl-γ-valerolactone), which indicated that the SPE as preparative technique clearly improved their analysis by UPLC–DAD–ESI–TQ MS by removing matrix interferences that suppress mass signals. Finally, there was a

group of compounds (4-*O*-methylgallic acid, syringic acid, and 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid) whose process efficiency was <85 %; these compounds were retained in the SPE cartridges and were incompletely recovered, which was a serious limitation for the application of this preparative technique to fecal samples.

These results suggest that due to the large structural diversity of the phenolic compounds present in fecal samples, the application of SPE would improve detection of certain phenolic metabolites (such as vanillic acid and 5-(3′,4′,-dihydroxyphenyl)- and 5-hydroxyphenyl-γ-valerolactone), but detection of other compounds (such as 4-*O*-methylgallic acid, syringic acid, and 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid) would be limited. Therefore, as a compromise solution and taking in mind the cost and labor associated to the SPE extraction, we decided not to use this preparative technique in the analysis of fecal phenolic metabolites presented hereafter.

Determination of Phenolic Metabolites in a Randomized Set of Fecal Samples

Analysis of the basal profile of phenolic metabolites in human feces appears to be a complementary approach to study phenolic metabolism by gut microbiota and physiological action

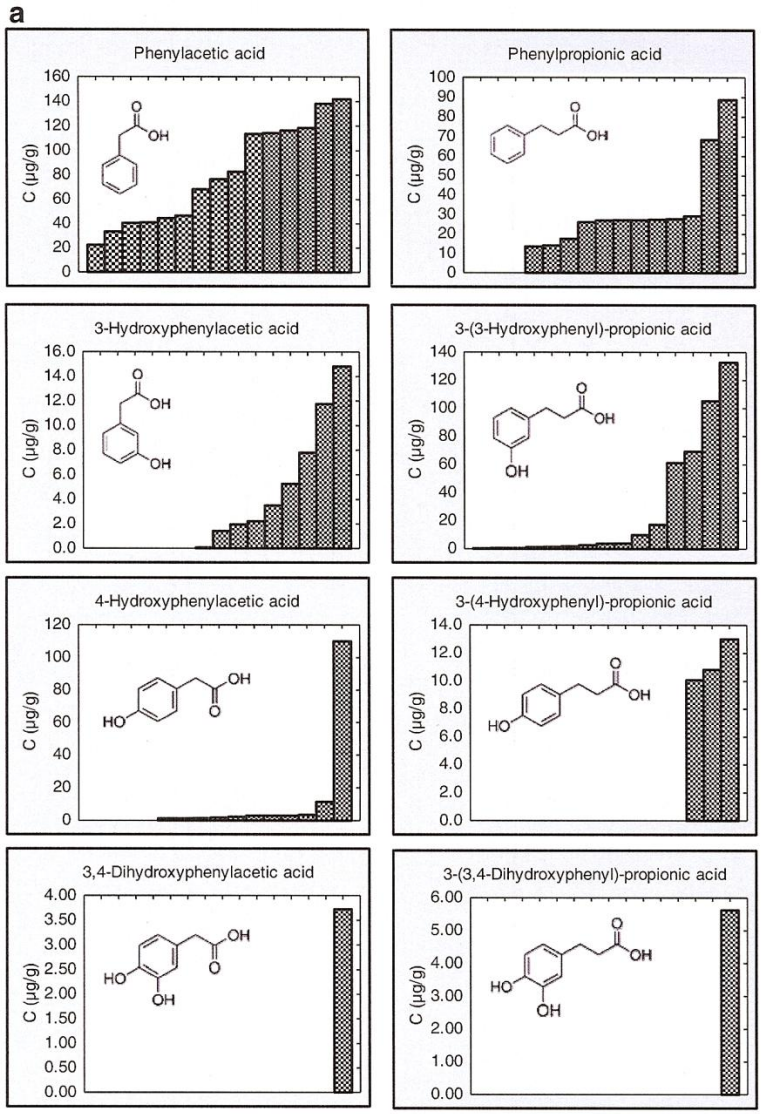

Fig. 1 Inter-individual distribution of the concentration of phenylpropionic and phenylacetic acids (**a**), benzoic acids (**b**) and cinnamic, mandelic and 4-hydroxy-5-(phenyl)-valeric acids (**c**) found in the randomized set of human fecal samples (n=15)

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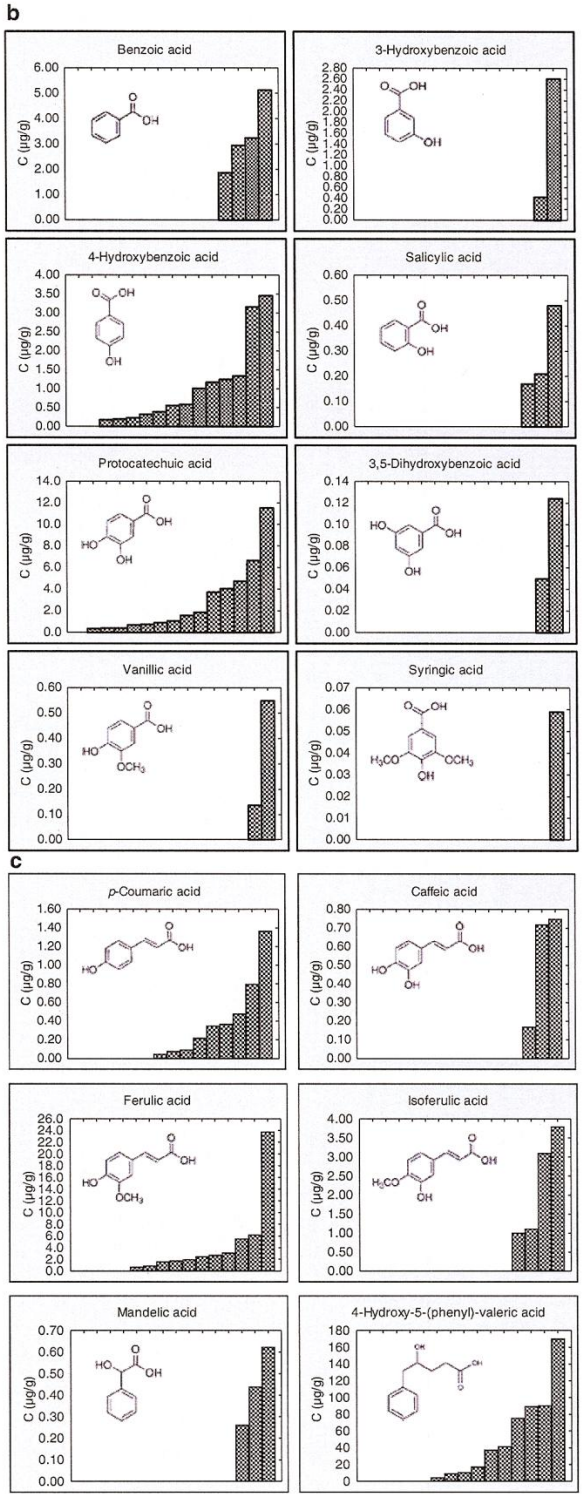

Fig. 1 (continued)

of polyphenols on microbiota. In order to improve knowledge about the basal content of phenolic metabolites in human feces, we determined the content of these metabolites in a randomized set of samples (n=15) from healthy volunteers. Based on the results reported above, the corresponding fecal solutions were not subjected to SPE extraction prior to analysis by UPLC–DAD–ESI–TQ MS.

Figure 1 shows the graphics of inter-individual distribution for each phenolic metabolite detected in the set of fecal samples. In total, 22 phenolic metabolites were detected but only three of them (phenylacetic acid, 3-(3-hydroxyphenyl)-propionic acid and protocatechuic acid) were found in all of the fecal samples. Among the microbial metabolites detected, fermentation end products and catabolites of flavan-3-ols were the most abundant, emphasizing the phenylacetic acid followed by 3-(3-hydroxyphenyl)-propionic acid, phenylpropionic acid, and 4-hydroxyphenylacetic acid which agreed with the data about the baseline profile of basal fecal samples described in the literature (Touriño et al. 2011; Jenner et al. 2005; Sánchez-Patán et al. 2011). Moreover, the detection and identification of 4-hydroxy-5-(phenyl)-valeric acid which has not been reported previously in human feces is an important finding given that it is a characteristic compound of the first steps of the microbial degradation of flavan-3-ols. Concerning concentrations, none of the phenolic acids detected exceeded concentrations >180 μg g^{-1}. Overall, large inter-individual variations among volunteers were observed, which was attributed to differences in the microbiota.

Conclusions

In this study, we have evaluated for the first time the effectiveness of an SPE methodology as a cleanup strategy prior to the analysis of phenolic acids in fecal samples. Oasis®HLB cartridges proved to be the most convenient sorbent for the extraction of phenolic acids of different hydroxylation pattern and side-chain length; however, dimeric procyanidins were largely retained in the cartridge and their process efficiency or recovery was low. This problem was also found for both monomers and dimers of flavan-3-ols after SPE of fecal samples spiked with the pool of phenolic standards, indicating that the technique was not appropriate for samples containing flavan-3-ols. On the other hand, further experiments indicated that the twofold diluted fecal matrix did not significantly affect the MS detection of the different standard phenolic acids. Moreover, SPE extraction of fecal samples collected after regular consumption of red wine by humans revealed a large difference in the process efficiency among the different type of phenolic metabolites and their MS detection could not be improved by increasing the volume of sample loaded on the cartridges. Considering all these findings and taking into account the cost and labor associated to this technique, we declined to use SPE as preparative technique for the analysis of fecal phenolic metabolites. Nevertheless, from our point of view, these results may be useful for anybody trying to develop general analytical procedures for the analysis of phenolic metabolites implying compounds of great chemical structure diversity. Finally, the paper reports valuable data about the content of phenolic metabolites in feces of healthy humans following a normal diet, confirming large qualitative and quantitative differences among individuals.

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Conflicts of Interest Irene Muñoz-González declares that she has no conflict of interest. Fernando Sánchez-Patán declares that he has no conflict of interest. Ana Jiménez-Girón declares that she has no conflict of interest. Carolina Cueva declares that she has no conflict of interest. María Monagas declares that she has no conflict of interest. Pedro J. Martín-Álvarez declares that he has no conflict of interest. M. Victoria Moreno-Arribas declares that she has no conflict of interest. Begoña Bartolomé declares that she has no conflict of interest.

Compliance with Ethics Requirements All procedures followed were in accordance with the ethical standards of the Ethics Committee of Clinical Investigation of the CSIC (Spain). Informed consent was obtained from all patients who are included in the study.

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<u>Publicación 5.1.2</u> Perfil de metabolitos fenólicos de origen microbiano en heces humanas, tras el consumo moderado de vino tinto

Profiling of Microbial-Derived Phenolic Metabolites in Human Feces after Moderate Red Wine Intake

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Resumen:

En el presente trabajo, se realizó un estudio de intervención en humanos, controlado y aleatorizado, que incluyó a 41 voluntarios sanos (33 casos y 8 controles), con el objetivo de evaluar los cambios en el perfil de metabolitos fenólicos de origen microbiano tras un consumo moderado de vino tinto (250 mL / día) durante 4 semanas. El análisis por UPLC-DAD-ESI-TQ MS permitió la identificación de 35 metabolitos, 10 de los cuales (principalmente ácidos benzoicos y 4-hidroxivaléricos) aumentaron significativamente (p<0,05) tras la ingesta de vino. Asimismo, el contenido total de metabolitos fenólicos fue significativamente (p<0,05) mayor en las muestras fecales tras la ingesta de vino (625 ± 380 μg/g de heces) respecto a las muestras basales (358 ± 270 μg/g de heces), y permitió realizar una clasificación tentativa de los voluntarios distribuidos en 3 grupos: bajo, medio y alto (<500, 500-1000, y >1000 μg/g de heces, respectivamente). Estos resultados sugieren que existe una gran variabilidad en la capacidad de la microbiota intestinal para metabolizar los polifenoles del vino, como se ha observado en el caso de otros polifenoles procedentes de diferentes fuentes.

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Profiling of Microbial-Derived Phenolic Metabolites in Human Feces after Moderate Red Wine Intake

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Supporting Information

ABSTRACT: A controlled and randomized trial study involving 41 healthy volunteers (33 intervention and 8 control subjects) was performed in order to establish changes in the microbial-derived phenolic metabolite profile of feces after moderate consumption of red wine (250 mL/day, 4 weeks). Out of the 35 phenolic metabolites identified, 10 compounds (mainly benzoic and 4-hydroxyvaleric acids) showed statistically significant increases ($P < 0.05$) after the wine intake. Also, the total phenolic metabolites content was significantly ($P < 0.05$) higher in the samples after the wine intake (625 ± 380 μg/g feces) in comparison to the samples before (358 ± 270 μg/g feces), and a tentative distribution of the volunteers into three groups could be established: <500, 500−1000, and >1000 μg/g feces. These results suggest that a different gut microbial capacity to metabolize wine polyphenols exists among the human population, as observed for polyphenols from other sources.

KEYWORDS: *red wine polyphenols, microbial metabolites, phenolic acids, feces, UPLC-ESI-MS/MS*

INTRODUCTION

The biological effects that phenolic compounds, including red wine polyphenols, exert at gut level, including anti-inflammatory activity, modulatory effects on the gut microbiota composition, and interaction with cells, among others, are a topic of current interest. It has become evident that these beneficial effects seem to be due more to phenolic metabolites formed in the gastrointestinal tract, mainly derived from the action of gut bacteria, rather than to the original forms found in food.[1,2] Therefore, there is a need to ascertain the phenolic profile and content in intestinal fluids, and, particularly, how they can be modified by diet. Given the difficulty in performing biopsies or any other intestinal fluid collection, a first approach can be to determine the phenolic profile in human feces.[3]

Polyphenols in wine comprise both flavonoid and nonflavonoid compounds, their molecular weight ranging from phenolic acids to highly polymerized proanthocyanidins. Flavonoids are more abundant in red wine and include flavan-3-ols and anthocyanins, as well as flavonols, flavanonols, and flavones, in lower proportions. The main nonflavonoids are phenolic acids (hydroxybenzoic and hydroxycinnamic acids), as well as other phenolic derivatives such as stilbenes. The intestinal metabolism of phenolic compounds starts in the epithelial cells with the hydrolysis, by intestinal β-glucosidases, of the glycosylated polyphenols such as anthocyanins, flavonols, and stilbenes and the subsequent absorption of the aglycon forms.[1,2] By contrast, monomeric flavanols and, in a lesser proportion, procyanidins dimers can be absorbed directly in the small intestine. Once absorbed, these polyphenols are first metabolized in the small intestine and then in the liver by phase II enzymes into methyl, glucuronide, or sulfate conjugates (phase II metabolites), which are preferentially excreted in the bile.[4] Other wine polyphenols, mainly oligomers with a degree of polymerization >3 and polymeric flavanols (proanthocyanidins or condensated tannins), hydroxycinnamic esters, and rhamnose-conjugated polyphenols, are not absorbed in their native forms. These compounds, together with phase II metabolites that reach the colon by enterohepatic recirculation, are catabolized by the colonic microbiota before their absorption.[5] Colonic catabolism involves the formation of simple phenols, phenolic and aromatic acids, and lactones with different degrees of hydroxylation and side-chain length, with often altered bioactivities compared to the parent compound,[6] that could be further absorbed and subsequently submitted to intestinal and hepatic metabolism by phase II enzymes.[1,7] Phenolic metabolites are excreted in urine and feces. So far, several human intervention studies have been carried out in order to study the colonic metabolism of wine polyphenols, reflected in plasma and urine.[8,9] In relation to feces, most of the knowledge acquired on the fecal profile of microbial metabolites comes from culture samples collected in in vitro fermentation studies.[10−13] To our knowledge, only a "pilot" study ($n = 8$ volunteers) has been conducted to ascertain the contents of phenolic metabolites in human feces after wine consumption.[14]

The study of biological responses due to a dietary intake of polyphenols cannot be managed without taking into consideration polyphenols−microbiota interactions. However, knowledge about the enzymatic activities/microbial population responsible for the degradation of polyphenols is still scarce.[2] But what is widely observed is the great interindividual variability in urine phenolic metaboloma after polyphenol-rich dietary interventions,[15−17] which has been attributed to

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dissimilarities in the populations of colonic bacteria. A first example of the different human capacity to metabolize polyphenols, as reflected in urine samples, was seen with isoflavones. In an intervention study with healthy premenopausal women (n = 60) who received soy-extract isoflavones, only some of the volunteers were able to metabolize daidzein to its active metabolite equol, which was attributed to differences in the composition of the intestinal microflora.[15] In addition, large interindividual variations were found in the equol-producing group, which was divided into two subgroups according to their ability to excrete more potent metabolites: high (approximately 25% of the volunteers) and low equol excretors.[15] Another example of the different human capacity to metabolize polyphenols refers to urolithins, microbial derived metabolites from ellagitannins. After an intervention with fresh strawberries and strawberry purée, urolithin A was found in the urine of all the volunteers (n = 20), but only three of them were found to produce and excrete urolithin B.[17] Moreover, volunteers were classified into three subgroups according to their levels of total urolithin A glucuronide excretion in urine.[17] A particular distribution of the volunteers according to their phenolic-metabolizing capacity was also suggested by Bolca et al.[18] in a dietary intervention (n = 150) with soymilk, soy germ, or hop. From their metabolite concentration in urine after the intervention, volunteers were phenotyped as poor, moderate, and strong producers of equol (derived from daidzein) or 8-prenylnaringenin (derived from isoxanthohumol).[18] Therefore, it is assumed that differences in human colonic microbiota lead to different polyphenol-metabolizing phenotypes, or "metabotypes", that in turn would govern the health effects of dietary polyphenols.[19] However, intervention/clinical studies with a large number of subjects are needed to establish these potential relationships between the consumption of foods rich in polyphenols and the production of phenolic metabolites.

To get a deeper understanding of the effects of wine consumption on human health, in the present work, a controlled and randomized trial study involving 41 healthy volunteers (33 intervention and 8 control subjects) was conducted in order to establish the changes in the phenolic metabolite profile of feces after the consumption of red wine polyphenols. Intervention was preceded by a 2-week baseline period with a restriction of wine and polyphenol consumption, and it was followed by a 4-week intervention period during which subjects consumed 250 mL of red wine per day. The changes in the fecal phenolic metabolites were determined using UPLC-ESI-MS/MS analysis and subjected to different statistical approaches. Our work has resulted in some improvements in the analysis of phenolic metabolites in feces by UPLC-ESI-MS/MS, invaluable data on the basal levels of phenolic metabolites in feces, in an assessment of significant changes in the contents and profile of phenolic metabolites in feces after wine consumption, and in a tentative attempt to distribute the population based on their capacity to metabolize wine polyphenols.

MATERIALS AND METHODS

Chemicals and Phenolic Standards. Acetic acid was purchased from Scharlau (Scharlau, Barcelona). Acetonitrile (HPLC grade) was purchased from Labscan (POCH S.A, Gliwice, Poland). Ultrapure water was obtained using a Milli-Q system (Waters Millipore, Milford, MA).

Phenolic standards used in this study and corresponding to mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, anthocyanins, flavan-3-ols, flavonols, and stilbenes were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), Phytolab (Vestenbergsgreuth, Germany), or Extrasynthèse (Genay, France). The standards 5-(3′,4′,-dihydroxyphenyl)-γ-valerolactone and 5-(4′-hydroxyphenyl)-γ-valerolactone were previously synthesized.[20] The compound 4-hydroxybenzoic-2,3,5,6-d_4 acid, used as internal standard, was purchased from Sigma-Aldrich Chemical Co.

For the analysis of phenolic metabolites by UPLC-ESI-MS/MS, a stock solution of phenolic standars in acetonitrile/water (1:4, v/v) was prepared by weighting individual compounds to achieve different concentrations (25, 50, 100, 200, and 500 μg/mL), depending on their response in ESI-MS/MS.[3] Dilutions of the stock solution were prepared and used in the generation of the calibration curves: 15 different concentration levels from 2- to 20 000-fold dilutions of the initial pool solution were injected in triplicate (Table 1-OSM, Supporting Information).

Red Wine. The red wine used in this study was a young red wine (var. Pinot Noir, vintage 2010), kindly provided by Bodegas Miguel Torres S.A. (Catalonia, Spain). The wine was elaborated following the winery's own winemaking procedures and was selected because of its relatively high phenolic content: total polyphenols = 1758 mg of gallic acid equiv/L, total anthocyanins = 447 mg of malvidin-3-O-glucoside/L, and total catechins = 1612 mg of (+)-catechin/L. The antioxidant capacity of the wine measured as ORAC (oxygen radical absorbance capacity) was 35.5 mmol of Trolox equiv/L.

Human Intervention Study Design. A total of 41 healthy volunteers (22 women and 19 men; age range 20−65 years) were recruited. The participants were not suffering from any disease or intestinal disorder and were not receiving antibiotics or any other medical treatment for at least 6 months before the start of the study or during the study (including the washout period). All the participants were fully informed about the study and gave written informed consent.

A randomized and controlled 4-week intervention study involving 33 volunteers was performed in parallel to an observational study (no intervention) for 8 volunteers (control group).

For both intervention and observational studies, volunteers followed an initial washout period of 2 weeks (baseline) during which they did not consume any wine or any other alcoholic beverage and followed a low-polyphenols diet. After this period, the intervention group consumed 250 mL of red wine/day (equivalent to a dose of ~450 mg of total polyphenols/day) divided into two doses, during 4 weeks. During this latter period, participants also maintained the restrictions for any other alcoholic beverages and followed a low-polyphenols diet. The control group followed the same pattern, with the exception that no wine was ingested during this 4-week period. Each participant provided samples of feces at two points: (a) after the washout period and (b) at the end of the study. Feces were immediately frozen and stored at −80 °C awaiting analysis.

Preparation of Fecal Solutions. For the preparation of fecal solutions, samples were thawed at room temperature and weighted (1.0 g) in 15 mL sterile conical tubes. Ten milliliters of sterile saline solution (NaCl 0.9%, Fresenius Kabi, Spain) spiked with the internal standard (IS) was added, vortexed, and centrifuged (10 min, 10 000 rpm, 4 °C) twice. The supernatant (fecal solution) was filtered (0.22 μm) and diluted with acetonitrile (1:4, v/v, acetonitrile/fecal solution). Saline solution contained 3.125 μg/mL of IS to achieve a final concentration of 2.5 μg/mL, and 2.0 μL of sample was injected onto the chromatographic system. Each analysis was realized in duplicate.

Analysis of phenolic metabolites. Phenolic metabolites were analyzed using an UPLC-ESI-MS/MS following a previously reported method.[3] The liquid chromatographic system was a Waters Acquity UPLC (Milford, MA) equipped with a binary pump, an autosampler thermostatted at 10 °C, and a heated column compartment (40 °C). The column employed was a BEH-C18, 2.1 × 100 mm and 1.7 μm particle size from Waters (Milford, MA). The mobile phases were 2% acetic acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient program was as follows: 0 min, 0.1% B; 1.5 min, 0.1% B;

11.17 min, 16.3% B; 11.5 min, 18.4% B; 14 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B. Equilibrium time was 2.4 min, resulting in a total runtime of 18 min. The flow rate was set constant at 0.5 mL/min and injection volume was 2 μL.

The LC effluent was pumped to an Acquity TQD tandem quadrupole mass spectrometer equipped with a Z-spray electrospray ionization (ESI) source operated in negative polarity mode. The ESI parameters were set as follows: capillary voltage, 3 kV; source temperature, 130 °C; desolvation temperature, 400 °C; desolvation gas (N_2) flow rate, 750 L/h; cone gas (N_2) flow rate, 60 L/h. The ESI was operated in negative ionization mode. For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific to each compound. The MS/MS parameters (cone voltage, collision energy and MRM transition) of the 60 phenolic compounds targeted in the present study (mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, 4-hydroxyvaleric acids, and valerolactones) were previously reported.[14] All metabolites were quantified using the calibration curves of their corresponding standards, except for 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric, 4-hydroxy-5-(3′-hydroxyphenyl)valeric, and 4-hydroxy-5-phenylvaleric acids, which were quantified using the calibration curves of 3-(3,4-dihydroxyphenyl)propionic, 3-(3-hydroxyphenyl)propionic, and propionic acids, respectively. 5-(3′-Hydroxyphenyl)-γ-valerolactone was quantified using the calibration curve of 5-(4′-hydroxyphenyl)-γ-valerolactone. Data acquisition and processing were realized with MassLynx 4.1 software.

Analysis of Wine Phenolic Compounds. Analysis of both anthocyanin and nonanthocyanin phenolic compounds present in the wine used in this study was carried out by UPLC-ESI-MS/MS, using the equipment reported in the previous section. For the analysis of anthocyanins, the gradient consisted of A (water/formic acid; 90:10, v/v) and B (acetonitrile) applied as follows: 0–1 min, 5–15% B; 1–5.25 min, 15–24% B; 5.25–5.88 min, 24–100% B; 5.88–7.05 min, 100–5% B; 7.05–9.38 min, 5% B. The ESI parameters were the same as those reported above for the analysis of phenolic metabolites, but ESI was operated in positive ionization mode. The MRM transitions used for the detection of anthocyanins were cyanidin-3-*O*-glucoside (449 > 287), delphinidin-3-*O*-glucoside (465 > 303), petunidin-3-*O*-glucoside (479 > 317), peonidin-3-*O*-glucoside (463 > 301), malvidin-3-*O*-glucoside (493 > 331), cyanidin-3-*O*-(6″-*O*-acetyl)glucoside (491 > 287), delphinidin-3-*O*-(6″-*O*-acetyl)glucoside (507 > 303), petunidin-3-*O*-(6″-*O*-acetyl)glucoside (521 > 317), peonidin-3-*O*-(6″-*O*-acetyl)glucoside (505 > 301), malvidin-3-*O*-(6″-*O*-acetyl)-glucoside (535 > 331), cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside (595 > 287), delphinidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside (611 > 303), petunidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside (625 > 317), peonidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside (609 > 301), malvidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside (639 > 331). Cone voltage and collision energy were set at 35 and 20 V, respectively, for all compounds. All individual anthocyanins were quantified using the external calibration curve of malvidin-3-*O*-glucoside. Data acquisition and processing was carried out using MassLynx 4.1 software.

For the analysis of nonanthocyanins, the gradient and the ESI parameters were the same as those reported above for the analysis of phenolic metabolites. The ESI was also operated in negative ionization mode. The MRM transitions used for the detection of flavan-3-ols were catechin and epicatechin (289 > 245), epicatechin-3-*O*-gallate (441 > 289), procyanidin dimers (B1, B2, B3, B4, B5, and B7) (577 > 289) and procyanidins trimers (C1 and others) (865 > 577). The MRM transitions used for the detection of other wine phenolic compounds were quercetin (301 > 151), kaempferol (285 > 93), myricetin (317 > 179), quercetin-3-*O*-glucoside (463 > 300), quercetin-3-*O*-galactoside (463 > 300), resveratrol (227 > 185), piceid (389 > 227), tryptophol (160 > 130), coutaric acid (295 > 163), and caftaric acid (311 > 179). In the absence of commercial standards, quantification of procyanidin B3, B4, B5, and B7 was carried out using the external calibration curve of procyanidin B1, and that of procyanidin trimers was based on procyanidin C1. Piceid was quantified using the external calibration curve of resveratrol. Coutaric acid and caftaric acid were quantified using the external calibration curves of *p*-coumaric acid and caffeic acid, respectively.

In addition, the wine was subjected to the analysis of phenolic metabolites as reported above to determine the content of benzoic acids, phenols, phenylacetic acids, phenylpropionic acids, and cinnamic acids. In all cases, analysis of wine (diluted 1:4, v/v, wine/water) was carried out in triplicate.

Statistical Analysis. The following statistical methods were used for the data analysis: *t* test for independent samples and its corresponding nonparametric Mann–Whitney test to evaluate differences in the means of the basal content of phenolic acid metabolites in feces between the control and intervention groups; *t* test for dependent samples and its corresponding nonparametric Wilcoxon matched-pairs test to evaluate differences in the means of the content of phenolic acid metabolites in feces before and after the 4-week study period, for both the control and intervention groups; the nonparametric Kruskal–Wallis test to compare the means of the content of phenolic metabolites between three groups of volunteers; and the Shapiro–Wilk test to verify the normal distribution of the data. A value of $P = 0.05$ was fixed for the level of significance of the tests. The STATISTICA program for Windows, version 7.1 (StatSoft. Inc. 1984–2006, www.statsoft.com) was used for data processing.

RESULTS AND DISCUSSION

Improvement of the Analysis of Fecal Solutions by UPLC-ESI-MS/MS: Use of Internal Standard Calibration. The ultrahigh-pressure LC separation method used in this work was adopted from a previous method developed in our laboratory.[3] In this method, fecal solutions (1:10 w/v, feces/saline solution) were 2-fold diluted prior to injection onto the UPLC system. Under these conditions, no significant differences were observed in the quantification of microbial phenolic metabolites between external and internal standard calibration methods.[3] To increase detectability of phenolic metabolites, a solid-phase extraction (SPE) procedure was further applied as a preparative sample technique prior to UPLC analysis.[21] However, although detection of certain phenolic metabolites was improved, the procedure failed in the recovery of other phenolics of interest, which led us to discard SPE as a preparative technique in the analysis of fecal phenolic metabolites by UPLC-ESI-MS/MS.[21] In this work, detectability and quantification of phenolic metabolites in feces have been improved by reducing the dilution factor of the fecal solution and by using an IS. Thus, different dilution factors of fecal solutions were tested and ion suppression-enhancement for the IS (4-hydroxybenzoic-2,3,5,6-d_4 acid) was studied by comparing its MS response in fecal solutions with respect to its response in acetonitrile/water (1:4, v/v), at a concentration value of 2.5 μg/mL (results not shown). A compromised dilution factor of 1.25 (4:1, v/v, fecal solution/acetonitrile) was adopted in order to obtain the lowest matrix effect assuring the detectability of the compounds of interest. The instrumental limits of detection (LOD) and quantification (LOQ) followed the criterion of signal-to-noise ratio (S/N) ≥3 and ≥8, respectively, and the calibration curves were determined by injection of the pool standard dilutions (Table 1-OSM, Supporting Information). Also, the accuracy and precision of the method was calculated by using the 1:250 dilution of the initial pool solution. The accuracy for all compounds [expressed as 100 × (mean observed concentration)/(added concentration)] using the internal standard calibration method was realized by performing three different injections/day and ranged from 80 to 113% (Table 1-OMS, Supporting Information). The precision (interday assay, $n = 5$), expressed

Table 1. Mean Values and Standard Deviations (mg/L) of the Individual Phenolic Compounds of the Red Wine Used in this Study

compd	concn	compd	concn
benzoic acids		flavan-3-ols and others	
gallic acid	27.30 ± 0.20	(+)-catechin	51.60 ± 1.70
protocatechuic acid	3.88 ± 0.01	(−)-epicatechin	34.90 ± 2.90
3-*O*-methylgallic acid	1.06 ± 0.06	procyanidin B1	79.10 ± 0.90
4-hydroxybenzoic acid	0.57 ± 0.01	procyanidin B2	44.70 ± 0.60
vanillic acid	1.85 ± 0.03	procyanidin B3	16.00 ± 1.00
syringic acid	2.30 ± 0.13	procyanidin B4	12.90 ± 0.30
benzoic acid	1.14 ± 0.06	procyanidin B5	2.67 ± 0.01
salicylic acid	0.21 ± 0.01	procyanidin B7	5.75 ± 0.15
		procyanidin C1	14.00 ± 0.40
phenols		other trimers	7.96 ± 1.05
phloroglucinol	0.33 ± 0.03		
tyrosol	31.40 ± 1.40	flavonols	
dihidroxiphenylpropan-2-ol	0.30 ± 0.04	quercetin	1.92 ± 0.01
		myricetin	0.70 ± 0.028
phenylacetic and phenylpropionic acids			
4-hydroxy-3-methoxyphenylacetic acid	0.01 ± 0.01	anthocyanins	
3-(3,4-dihydroxyphenyl)-propionic acid	0.16 ± 0.02	delphinidin-3-*O*-glucoside	2.58 ± 0.11
		cyanidin-3-*O*-glucoside	0.76 ± 0.04
cinnamic acids		petunidin-3-*O*-glucoside	4.06 ± 0.13
caffeic acid	6.97 ± 0.26	peonidin-3-*O*-glucoside	18.90 ± 2.00
p-coumaric acid	1.39 ± 0.02	malvidin-3-*O*-glucoside	36.70 ± 3.40
ferulic acid	0.22 ± 0.02	delphinidin-3-*O*-(6″-acetyl)glucoside	0.14 ± 0.01
coutaric acid	8.64 ± 0.01	cyanidin-3-*O*-(6″-acetyl)glucoside	0.08 ± 0.01
caftaric acid	4.98 ± 0.33	petunidin-3-*O*-(6″-acetyl)glucoside	0.15 ± 0.01
		peonidin-3-*O*-(6″-acetyl)glucoside	0.87 ± 0.07
stilbenes		malvidin-3-*O*-(6″-acetyl)glucoside	1.17 ± 0.03
resveratrol	7.12 ± 0.29	delphinidin-3-*O*-(6″-*p*-coumaroyl)glucoside	0.05 ± 0.01
		cyanidin-3-*O*-(6″-*p*-coumaroyl)glucoside	0.07 ± 0.01
		petunidin-3-*O*-(6″-*p*-coumaroyl)glucoside	0.07 ± 0.01
		peonidin-3-*O*-(6″-*p*-coumaroyl)glucoside	1.09 ± 0.07
		malvidin-3-*O*-(6″-*p*-coumaroyl)glucoside	0.94 ± 0.04

as the relative standard deviations (% RSD), was lower than 15% in all cases (Table 1-OSM, Supporting Information).

Phenolic Metabolic Profile in Human Feces after Moderate Red Wine Intake. Phenolic compounds determined in the red wine consumed in this study included benzoic acids, phenols, phenylacetic and phenylpropionic acids, cinnamic acids, stilbenes, flavan-3-ols, flavonols, and anthocyanins, as determined by UPLC-ESI-MS/MS (Table 1). Of special note was the wine's richness in monomeric and oligomeric flavan-3-ols (270 mg/L).

Fecal samples from each volunteer before and after wine intake were analyzed by the described UPLC-ESI-MS/MS methods. Within the 60 targeted metabolic phenolics, a total of 35 compounds were quantified, although in a variable number of cases, for both control (after the 4-week observational period) and intervention groups (after the 4-week wine intervention period) (Table 2). Most of the metabolites were found in a higher number of volunteers after wine intake than before the intervention. This was especially noticeable for syringic acid, since it was quantified in only 4 samples before the wine intake and in 26 samples after the intervention. In a previous pilot study, only 22 phenolic metabolites were detected in feces after a wine intervention study (272 mL/day, 20 days),[14] which evidences either the improvements made on the method to enhance its sensitivity and/or the higher phenolic content of the wine used in the present study. No detectable amounts of the phenolic compounds presented in the wine (anthocyanins, flavan-3-ols, flavonols, or stilbenes) were found in the fecal samples. For a better understanding, mean values of phenolic metabolites concentration for the two groups (control and intervention groups) were calculated by excluding negligible cases (<LOQ) (Table 2). On the basis of these results, benzoic acid, phenylacetic acid, 3-(4′-hydroxyphenyl)propionic acid, 3-(3′-hydroxyphenyl)propionic acid, 3-phenylpropionic acid, and 4-hydroxy-5-phenylvaleric acid showed the highest concentrations for both groups before the 4-week study period. The mean values of phenolic compounds at baseline obtained in the present work were included within the concentration ranges previously reported in the literature,[22−27] in spite of the differences in methodology, number of volunteers, and sample preparation among studies. Table 2-OSM (Supporting Information) reports the basal concentrations in feces for most abundant phenolic metabolites reported in the literature, in comparison to the data reported in this work. Nevertheless, it is necessary to point out the high interindividual variability observed in the concentrations of fecal phenolic metabolites within each study, which evidence the difficulty in establishing reference values and the importance of carrying out these studies with a large group of volunteers, under controlled diets, in order to obtain reliable and consistent conclusions.

Table 2. Phenolic Metabolite Concentration in Human Feces for the Control and Wine Intervention Groups before and after Wine Intake

compd	metabolite concn[a] (μg/g feces)			
	control group (n = 8)		intervention group (n = 33)	
	before	after	before	after
mandelic acids				
3-hydroxymandelic acid	nd[b]	nd	1.77 (n = 1)[c]	1.77 ± 0.01 (n = 2)
benzoic acids				
gallic acid	2.05 ± 0.01 (n = 2)	2.02 ± 0.06 (n = 2)	2.36 ± 0.77 (n = 10)	2.19 ± 0.24 (n = 17)
3,5-dihydroxybenzoic acid	0.18 ± 0.21 (n = 8)	0.18 ± 0.13 (n = 7)	0.21 ± 0.16 (n = 26)	0.35 ± 0.21 (n = 32)
protocatechuic acid	0.99 ± 1.20 (n = 8)	1.14 ± 1.76 (n = 8)	0.79 ± 0.47 (n = 33)	1.25 ± 1.03 (n = 33)
3-*O*-methylgallic acid	0.627 (n = 1)	0.27 (n = 1)	0.50 ± 0.37 (n = 5)	0.37 ± 0.18 (n = 24)
4-hydroxybenzoic acid	1.15 ± 1.40 (n = 6)	0.90 ± 0.78 (n = 6)	0.74 ± 0.77 (n = 23)	0.69 ± 0.68 (n = 24)
4-*O*-methylgallic acid	nd	nd	1.15 ± 0.06 (n = 3)	1.24 ± 0.13 (n = 3)
3-hydroxybenzoic acid	0.95 ± 0.49 (n = 4)	0.59 ± 0.61 (n = 5)	0.63 ± 0.32 (n = 9)	1.04 ± 1.35 (n = 12)
vanillic acid	1.10 (n = 1)	0.98 ± 0.52 (n = 2)	1.08 ± 2.33 (n = 9)	1.12 ± 0.84 (n = 27)
syringic acid	0.42 (n = 1)	nd	0.77 ± 0.37 (n = 4)	1.84 ± 1.11 (n = 26)
benzoic acid	19.40 ± 15.00 (n = 8)	21.00 ± 22.50 (n = 8)	37.70 ± 25.50 (n = 33)	43.40 ± 35.90 (n = 33)
salicylic acid	0.18 ± 0.02 (n = 2)	0.38 ± 0.42 (n = 4)	0.92 ± 1.01 (n = 6)	0.69 ± 1.32 (n = 9)
phenols				
catechol/pyrocatechol	2.25 (n = 1)	nd	0.98 ± 0.25 (n = 3)	1.14 ± 0.55 (n = 6)
4-methylcatechol	nd	nd	0.97 ± 0.14 (n = 2)	1.78 (n = 1)
hippuric acids				
4-hydroxyhippuric acid	0.80 (n = 1)	0.33 (n = 1)	0.34 (n = 1)	0.35 (n = 1)
phenylacetic acids				
3,4-dihydroxyphenylacetic acid	4.77 ± 3.55 (n = 2)	2.28 (n = 1)	6.87 ± 10.04 (n = 5)	6.65 ± 8.16 (n = 7)
4-hydroxyphenylacetic acid	9.62 ± 13.50 (n = 6)	3.58 ± 3.32 (n = 7)	4.72 ± 7.62 (n = 31)	4.09 ± 4.99 (n = 31)
3-hydroxyphenylacetic acid	2.17 ± 1.22 (n = 7)	4.99 ± 5.80 (n = 8)	9.06 ± 8.19 (n = 29)	18.60 ± 20.10 (n = 33)
4-hydroxy-3-methoxyphenylacetic acid	0.40 (n = 1)	nd	4.60 ± 5.91 (n = 4)	6.29 ± 10.10 (n = 3)
phenylacetic acid	67.90 ± 39.20 (n = 8)	93.90 ± 59.70 (n = 8)	161.00 ± 141.00 (n = 33)	183.00 ± 167.00 (n = 32)
phenylpropionic acids				
3-(3′,4′-dihydroxyphenyl)-propionic acid	3.53 ± 2.46 (n = 5)	2.28 ± 0.61 (n = 4)	5.06 ± 7.62 (n = 13)	4.30 ± 5.09 (n = 10)
3-(4′-hydroxyphenyl)-propionic acid	30.70 ± 28.20 (n = 3)	12.70 (n = 1)	21.90 ± 15.60 (n = 5)	23.90 ± 17.70 (n = 7)
3-(3′-hydroxyphenyl)-propionic acid	17.60 ± 30.40 (n = 8)	12.30 ± 23.10 (n = 8)	48.30 ± 138.60 (n = 33)	48.00 ± 119.20 (n = 33)
3-phenylpropionic acid	30.10 ± 29.40 (n = 7)	30.00 ± 21.20 (n = 6)	62.40 ± 63.60 (n = 30)	81.60 ± 62.70 (n = 29)
valeric acids				
4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid	nd	1.46 ± 0.06 (n = 2)	1.42 ± 0.03 (n = 5)	1.65 ± 0.75 (n = 9)
4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid	0.12 ± 0.04 (n = 4)	0.16 ± 0.12 (n = 4)	0.12 ± 0.04 (n = 9)	0.12 ± 0.06 (n = 15)
4-hydroxy-5-phenylvaleric acid	12.90 ± 8.90 (n = 6)	18.70 ± 12.70 (n = 6)	30.20 ± 37.60 (n = 27)	241.00 ± 226.00 (n = 32)
valerolactones				
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	nd	2.64 (n = 1)	0.39 ± 0.66 (n = 5)	12.00 ± 11.80 (n = 5)
5-(3′-hydroxyphenyl)-γ-valerolactone	nd	nd	nd	24.30 ± 23.00 (n = 5)
5-(4′-hydroxyphenyl)-γ-valerolactone	nd	1.86 (n = 1)	11.80 ± 13.70 (n = 3)	5.12 ± 3.29 (n = 8)
cinnamic acids				
caffeic acid	2.03 ± 0.25 (n = 7)	2.08 ± 0.30 (n = 5)	2.13 ± 0.32 (n = 25)	2.07 ± 0.66 (n = 26)
p-coumaric acid	1.39 ± 0.20 (n = 8)	1.31 ± 0.04 (n = 8)	1.49 ± 0.35 (n = 30)	1.54 ± 0.34 (n = 32)
m-coumaric acid	nd	nd	0.33 ± 0.09 (n = 2)	0.34 ± 0.07 (n = 5)
ferulic acid	2.69 ± 2.15 (n = 6)	1.66 ± 0.19 (n = 7)	2.68 ± 2.30 (n = 30)	2.29 ± 1.35 (n = 28)
isoferulic acid	4.24 (n = 1)	nd	3.61 ± 0.21 (n = 4)	4.27 ± 1.03 (n = 3)

[a]Means and SD were calculated excluding negligible cases. [b]nd: not detected. [c]In parentheses is the number of cases considered for each compound.

Statistical analysis of data comprised both comparisons among the control and intervention groups before the 4-week study period (basal content) and comparisons before and after the 4-week study period within each group. For statistical comparison of means, all cases were considered, including negligible data (zero values). The t test for independent samples and its corresponding nonparametric Mann–Whitney test were realized to evaluate differences in the means of the basal content of phenolic acid metabolites in feces between the control and intervention groups. No significant differences (P > 0.05) were found in the content of phenolic metabolites between the two groups except for the 3-hydroxyphenylacetic acid (P < 0.05). However, when the sum of the concentration of all metabolites (total phenolic metabolite content) was considered, no statistically significant differences (P > 0.05) among groups were observed in this basal content (175 ± 98 and 358 ± 270 μg/g feces for the control and intervention groups, respectively). Hence, it can be concluded that the distribution of volunteers between the control and intervention groups was adequate.

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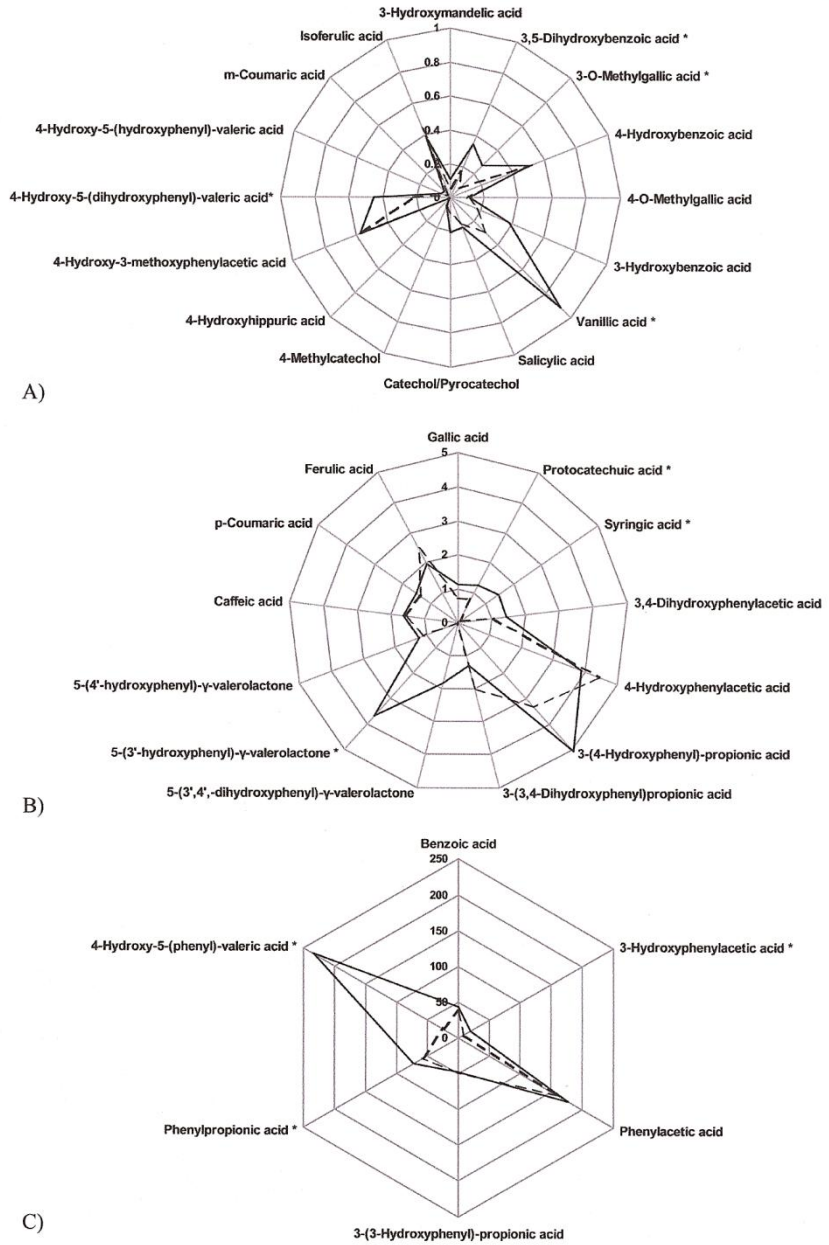

Figure 1. Phenolic metabolites in feces for the wine-intervention group (n = 33), before (dashed line) and after wine intake (solid line). (A) Metabolites with a content of (A) <1 μg/g, (B) 1−5 μg/g, and (C) 5−250 μg/g. *Mean values significantly different in concentration before and after wine intake (P < 0.05).

For the control group, the t test for dependent samples and the nonparametric Wilcoxon matched-pairs test showed no significant differences (P > 0.05) between samples before and after the 4-week study period, as expected. When the t test was applied to the intervention group, significant differences (P < 0.05) between samples before and after the wine intake were found for the content of 3,5-dihydroxybenzoic acid, protocatechuic acid, 3-*O*-methylgallic acid, vanillic acid, 3-hydroxyphenylacetic acid, syringic acid, 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid, and 4-hydroxy-5-phenylvaleric acid. Also, the total phenolic metabolites content was significantly higher in the samples after wine intake (625 ± 380 μg/g feces) compared to the samples before wine intake (358 ± 270 μg/g feces). Moreover, when the nonparametric Wilcoxon matched-pairs test was realized, aside from the previous compounds, significant differences were found for the content of 5-(3′-hydroxyphenyl)-γ-valerolactone and 3-phenylpropionic acid, whose data did not follow a normal distribution according to the Shapiro−Wilk test. To visualize changes in the fecal phenolic metabolite profile, radar diagrams including all

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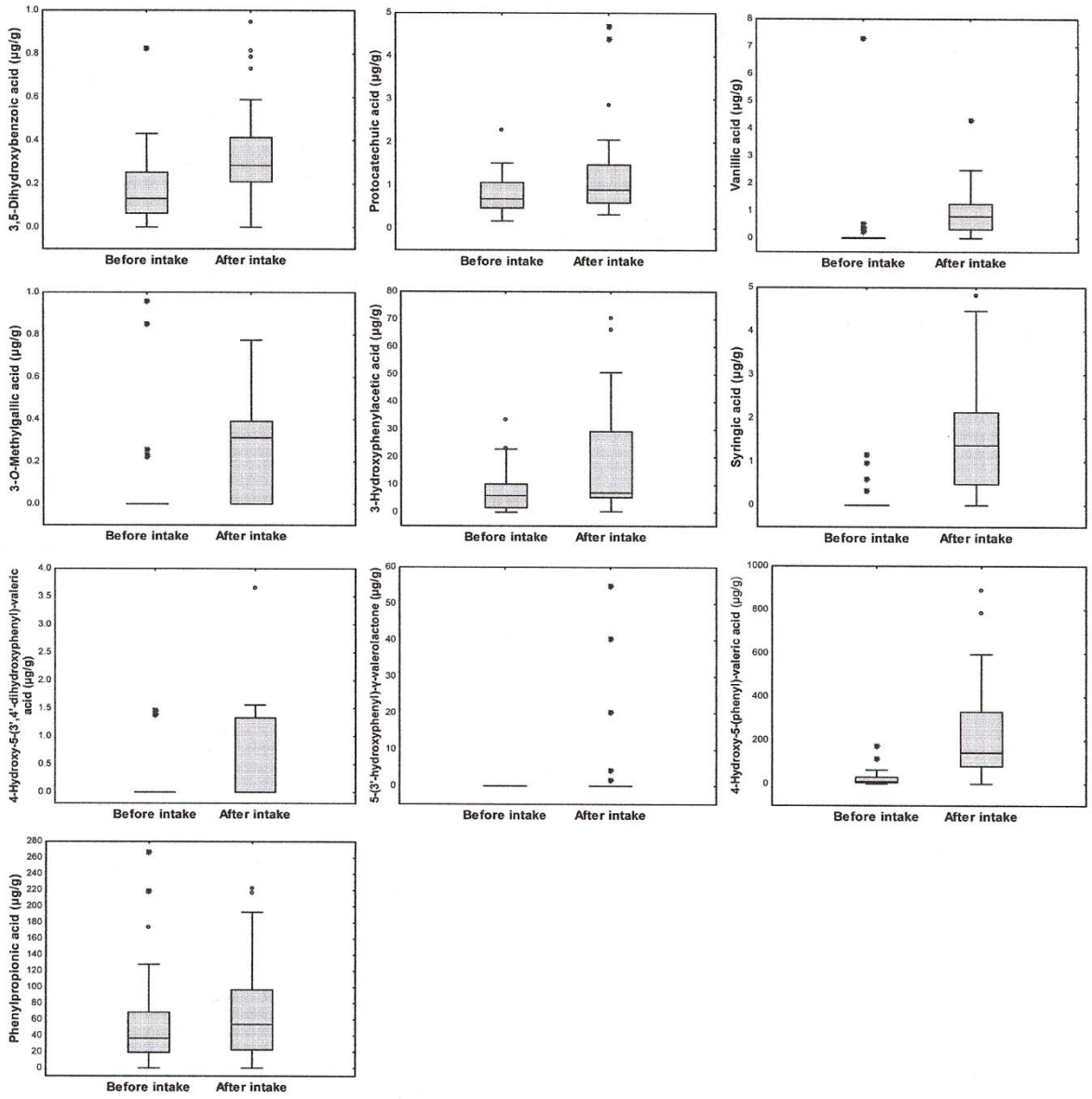

Figure 2. Box and whiskers plots (—, median; □, 25th–75th percentiles; |, nonoutlier range; ○, outliers; and *, extremes) of the phenolic metabolites found to be significantly different for the intervention group (n = 33) after wine intake.

metabolites detected in the fecal samples before (dashed line) and after wine intake (solid line) were constructed (Figure 1). Metabolites were classified into three groups according to their amount in fecal samples: (A) < 1 µg/g feces, (B) 1–5 µg/g feces, and (C) 5–250 µg/g feces. As can be seen, most of the compounds (n = 16) were found in low concentrations (<1 µg/g), with four of them showing a significantly different concentration level after wine intake (Figure 1). Three compounds among the metabolites with a concentration between 1 and 5 µg/g feces (n = 13) and three more metabolites among the metabolites with concentrations between 5 and 250 µg/g (n = 6) showed significant differences (P < 0.05) after intervention. Comparing the radar profiles between the fecal samples, before and after the wine intake, resulted in quite similar shapes for the three metabolite groups, with the exception of peaks corresponding to vanillic acid, 5-(3′-hydroxyphenyl)-γ-valerolactone, and 4-hydroxy-5-phenyl-valeric acid (quantified as propionic acid) in the fecal samples after the wine intake (Figure 1). Variability among volunteers is clearly shown in Figure 2, which displays the box and whiskers plots (median, 25th and 75th percentiles, nonoutlier range, outliers, and extremes) of the individual microbial metabolites mentioned above which showed significant differences (P < 0.05) before and after the red wine intervention. In general, the 25th–75th percentiles were greater for the samples after the wine intake than samples before the wine intake, indicating that

wine polyphenol metabolism enhances interindividual variability further still.

Distribution of the Volunteers Based on Their Total Phenolic Metabolite Content in Their Feces. In an attempt to distribute the volunteers by looking at their capacity to metabolize wine polyphenols, frequency histograms for the total phenolic metabolite content in human feces for both the control and intervention groups were constructed (Figures 3 and 4, respectively). For the control group (n = 8), the distribution of the volunteers was similar before and after the 4-week intervention period. The frequency histogram for the intervention group (n = 33) became wider and moved toward higher values after the 4-week wine intake (Figure 4). On the basis of these histograms, the volunteers were tentatively distributed into three groups: (a) volunteers with a total phenolic metabolite content <500 μg/g feces, (b) volunteers with a total phenolic metabolite content between 500 and 1000 μg/g feces, and (c) volunteers with a total phenolic metabolite content >1000 μg/g feces. The group with total phenolic metabolite content <500 μg/g feces included 27 volunteers (82% of the total) before the wine intake, but only 13 (39% of the total) after the intake. In contrast, the number of volunteers in the group exhibiting a total phenolic content of 500−1000 μg/g feces increased from 5 to 13 volunteers after the wine intake. The same was observed for the group of highest phenolic metabolite content (>1000 μg/g feces), the number of volunteers increasing from 1 to 7 after the wine intake (Figure 4).

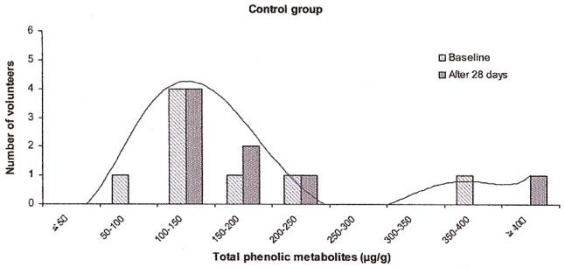

Figure 3. Distribution, by the total phenolic metabolites content, of the control group volunteers before and after the 28-day period.

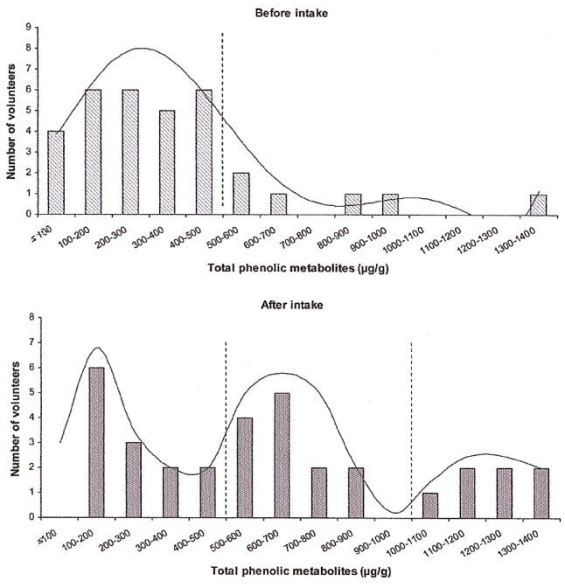

Figure 4. Distribution, by the total phenolic metabolites content, of the study group volunteers before and after the 28-day period of wine intake.

The means of the fecal contents of phenolic metabolites corresponding to the three groups of volunteers described above (<500, 500−1000, and >1000 μg/g feces) were compared after the 4-week wine intervention. The nonparametric Kruskal−Wallis test was applied once it was confirmed that the phenolic metabolite contents did not follow a normal distribution according to the Shapiro−Wilk test. Differences among the three groups (<500, 500−1000, and >1000 μg/g feces) were found for the contents of 3,4-dihydroxyphenylacetic acid, catechol/pyrocatechol, 3-(4-hydroxyphenyl)propionic acid, *m*-coumaric acid, 3-(3-hydroxyphenyl)propionic acid, phenylacetic acid, phenylpropionic acid, and 4-hydroxy-5-phenylvaleric acid, supporting the feasibility of the volunteer distribution proposed. As indicated in the Introduction, other authors have proposed classifying the human population into high, moderat,e and low metabolizers, taking into consideration the levels of a specific phenolic metabolite in urine after an intervention with a source rich in the corresponding precursors.[17,18] This paper shows that this different human capacity for metabolizing polyphenols is also reflected in the fecal metabolome, as seen with wine polyphenols. The volunteer classification made in this paper appears complex since it attempts to cover the global capacity of metabolizing wine polyphenols that encompass different phenolic groups. But even taking that into consideration, the volunteers could be tentatively classified into high, moderate, and low metabolizers based on the total phenolic content in feces after wine consumption.

On the other hand, to our knowledge, very few studies have reported changes in the content of fecal phenolic metabolites after a human intervention study.[25,28,29] Recently, Gill et al.[25] reported changes in the phenolic metabolite contents after an intervention study (n = 10) consisting of the intake of raspberry purée (200 g/day) during 4 days. From these published data in μg/mL, and considering an average fecal water content of 65%, we have expressed the means of fecal phenolic metabolites after this intervention as 145 μg/g for phenylacetic acid after raspberry purée, in comparison to the value of 183 μg/g reported in this paper after 4-weeks of wine intervention; 67.6 μg/g for 3-phenylpropionic acid, in comparison to the value of 81.6 μg/g reported in this paper; 20.8 μg/g for 3-(4′-hydroxyphenyl)propionic acid, in comparison to the value of 23.9 μg/g feces reported in this paper; 14.4 μg/g for 3-hydroxyphenylacetic acid, in comparison to the value of 18.6 μg/g reported in this paper; and 1.63 μg/g for 3,4-dihydroxyphenylacetic acid, in comparison to the value of 6.65 μg/g reported in this paper. Only the fecal contents of benzoic and isoferulic acids were significantly lower after the intervention with raspberry purée (200 g/day, 4 days) (2.83 and 0.411 μg/g, respectively) than after the red wine intervention (272 mL/day, 4 weeks) (43.4 and 4.27 μg/g, respectively), although finally concluding the consistency between both studies.

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In conclusion, this paper reports valuable data on the basal levels of phenolic metabolites in feces, which are in accordance to those reported in the literature. Although showing a great interindividual variability, these data may be helpful for the design of in vitro and cell culture studies to address the biological effects that polyphenols exert at the gut level. Also, it has been proven that the moderate consumption of red wine significantly promotes changes in the metabolic prolife and content in feces, with may be related to positive biological effects. In parallel to this work, we are conducting studies of characterization and metabolic activity of the fecal microbiota from the same human samples reported here, with the final aim to go deeper in the understanding of the polyphenol–microbiota interactions.

■ ASSOCIATED CONTENT

Supporting Information

Table 1-OSM shows retention times and MS/MS parameters for the investigated phenolic metabolites and Table 2-OSM lists the content of phenolic compounds in feces at baseline reported in literature. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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<u>Publicación 5.1.3</u> Perfil metabolómico en heces tras el consumo moderado de vino tinto en individuos sanos

Faecal Metabolomic Fingerprint after Moderate Consumption of Red Wine by Healthy Subjects

Ana Jiménez-Girón, Clara Ibáñez, Alejandro Cifuentes, Carolina Simó, **Irene Muñoz-González**, Pedro J. Martín Álvarez, Begoña Bartolomé, M.Victoria Moreno-Arribas. *Journal of Proteomic Research*, **2014** (*Enviado para su publicación).*

Resumen:

El metaboloma fecal contiene información acerca de los metabolitos que se encuentran en el intestino, de los cuales se puede obtener información muy útil acerca de la función metabólica de la microbiota intestinal. Los cambios en el perfil metabolómico en heces reflejan, entre otros, cambios en la composición y actividad metabólica de los microorganismos intestinales. Muchas de estas actividades metabólicas son aún desconocidas y están pobremente caracterizadas. En un esfuerzo por mejorar la comprensión acerca de los efectos fisiológicos que los compuestos fenólicos (incluyendo los polifenoles del vino tinto) ejercen a nivel intestinal, en este estudio *foodómico* se ha llevado a cabo una caracterización del metaboloma de heces humanas tras un consumo moderado de vino tinto por parte de sujetos sanos durante 4 semanas. Se desarrolló un método metabolómico no dirigido basado en el uso de UHPLC-TOF MS para lograr la máxima información acerca del perfil de metabolitos en 82 muestras fecales humanas. Tras el procesamiento de los datos y el análisis estadístico, se encontró que 37 metabolitos estaban relacionados con la ingesta de vino, de los cuales pudieron ser tentativa o completamente identificados 20 metabolitos, incluyendo: I) compuestos del vino, II) metabolitos microbianos derivados de los polifenoles del vino, y III) metabolitos endógenos y/o derivados de otras vías de nutrientes. Tras el consumo de vino, el metaboloma fecal fue fortificado en metabolitos derivados de flavan-3-oles. Además, fue relevante la regulación a la baja de la xantina y metabolitos derivados de la bilirrubina tales urobilinógeno y estercobilina tras el consumo moderado de vino. Hasta donde sabemos, este es el primer estudio del metaboloma fecal después de la ingesta de vino.

Abstract

Faecal metabolome contains information of the metabolites found in the intestine from which information about the metabolic function of the gut microbiota can be obtained. Changes in the metabolomic profile of faeces reflect, among others, changes in the composition and activity of the intestinal microorganisms. Many of these metabolisms are poorly characterized and understood. In an effort to improve our understanding on the biological effects that phenolic compounds (including red wine polyphenols) exert at the gut level, in this foodomic study we have undertaken a metabolome characterization of human faeces after moderate consumption of red wine by healthy subjects for 4 weeks. Namely, a non-targeted metabolomic approach based on the use of UHPLC-TOF MS was developed to achieve the maximum metabolite information of 82 human faecal samples. After data processing and statistical analysis, 37 metabolites were related to wine intake from which 20 could be tentatively or completely identified, including: I) wine compounds, II) microbial-derived metabolites of wine polyphenols, and III) endogenous metabolites and/or others derived from other nutrient pathways. After wine consumption, faecal metabolome was fortified in flavan-3-ols metabolites. Also, of relevance was the down regulation of xanthine and bilirubin-derived metabolites such urubilinogen and stercobilin after moderate wine consumption. As far as we know, this is the first study of the faecal metabolome after wine intake.

1 Introduction

Epidemiological and clinical studies have evidenced that regular and moderate wine consumption is linked to lower incidence of cardiovascular disease (CVD), hypertension, diabetes, and certain types of cancer[1,2]. More recently, some intervention studies have showed that red wine consumption may promote gut health by selective modulation of gut microbiota, through the antimicrobial or eventually

prebiotic-like properties of its components and/or metabolites derived from them[3]. Indeed, wine polyphenols are assumed to play a key role in all the health-promoting effects associated with moderate wine consumption. In general, these phytochemicals are known to be poorly absorbed in the small intestine, reaching the colon where they are catabolized by the gut microbiota to give rise to a great battery of phenolic metabolites that can be further absorbed and also secreted in the faeces[4].

The study of the effects derived from certain food intakes or diets is gaining increasing interest, with special emphasis on the improvement of our limited understanding of the roles of dietary components at molecular level (i.e., their interaction with genes, and their subsequent effect on proteins and metabolites). The application of omics technologies has brought about extraordinary opportunities to solve these complex issues, since they can provide new and important information about the biochemical, molecular and cellular mechanisms that underlie the beneficial or adverse effects of certain bioactive food components[5]. In this regard, a global methodology such as Foodomics, based on the combination of several omics platforms and data processing tools, is well suited to perform comprehensive evaluations of the health benefits of food intake[6,7]. Among omics technologies, metabolomics has recently emerged to complement the information obtained from genomics and proteomics in the field of nutrition research[8,9]. Metabolomics involves the analysis of small molecules or metabolites present in biological samples with the aim to study alterations in metabolism under different conditions. As already known, metabolome content of body fluids is an important source of information about dietary exposure[10]. Metabolomics studies will also help us to gain further insight into human metabolic pathways regarding their relationship with diet factors. Urinary and plasma metabolomes have been studied in a variety of nutritional intervention studies, such as, citrus[11], fruits and vegetables-rich diet[12], cocoa intake[13], apple consumption[14], nut ingestion[15], among the most recent published works. The influence of the gut microbiome and its interaction with the host is essential to understand nutrition and

metabolism[16]. Faecal samples (both faeces and faecal water), that are often used in analysis of gut-based disorders, are particularly complex samples, directly related to colonic microbiota and external factors. The intestinal lumen harbours a huge number of compounds formed during the metabolism of nutrients and xenobiotics (phase I, phase II and microbial metabolites), and many of these metabolites are excreted in faeces. In this sense, studying the faecal metabolome represents a potent strategy for understanding interactions between nutrients, the intestinal metabolism and the microbiota composition in health and disease[17].

Until now, few human nutritional intervention studies have been carried out to assess the metabolomic impact of wine consumption. The human metabolome in urine and plasma after a short-term intake (5 days) of red wine and grape polyphenols by healthy volunteers was assessed by a combination of GC-MS- and LC-MS/MS approaches[18]. Vazquez-Fresno et al.[19] applied an H-NMR-based metabolomic protocol to urine samples from subjects with cardiovascular risk factors after consumption of red wine, dealcoholized red wine and gin for 4 weeks. In relation to the faecal metabolome and wine intake, only Jacobs et al.[20] have attempted the H-NMR metabolite profiling in healthy volunteers who followed a polyphenols-reach diet (a mixture of grape juice and wine extract) for 4 weeks. With the aim to continue and look more deeply into the biological effects that phenolic compounds (including red wine polyphenols) exert at gut level, in this paper we have studied the metabolomic fingerprint of faecal samples from healthy volunteers after the consumption or red wine for 4 weeks. A liquid chromatography/time-of-flight mass spectrometry (LC-TOF MS) method has been developed to obtain relevant faecal markers of red wine consumption. Variations of the faecal metabolome upon this intervention with red wine have confirmed metabolic pathways of wine components -polyphenols in particular- in the human body, and have revealed new mechanisms of action of wine, especially in relation to gut microbiota.

2 Experimental Sections

2.1 Chemicals

All chemicals were of analytical grade. Formic acid was from Riedel-de Haën (Seelze, Germany). Acetonitrile and water were of MS grade and purchased from Labscan (Gliwice, Poland). A commercial standard mixture containing 42 low- molecular weight compounds (acidics, basics and neutrals, ABN) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used to assess instrument variability along the study. Commercial standards of xanthine, glutaric acid, benzoic acid, L-lysine, ascorbic acid, pyruvica acid, fumaric acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, γ-valerolactone, 4-methoxyphenylacetic acid, 3-(4-hydroxyphenyl)-propionic acid, 3-(3-hydroxyphenyl)-propionic acid, 3-(3,4-dimethoxyphenyl)-propionic acid, L-ornithine monohydrochloride, 2-methyl amino benzoic acid and methyl 2-aminobenzoate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and used for identification purposes. Stercobilin and urobilinogen were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA) and also used for identification purposes.

2.2 Red wine

A young red wine (Pinot Noir, vintage 2010) with a total phenolic content of 1758 mg of gallic acid equivalents/L, total anthocyanins = 447 mg of malvidin-3-O-glucoside/L and total catechins = 1612 mg of (+)-catechin/L was selected for the intervention study. Ethanol, pH, total acidity and volatile acidity were determined in the wine according to the International methods of the OIV (International Organization of Vine and Wine, 1990). The resulting properties were as follows: the wine pH was 3.52; alcohol degree: 13.8 % v/v, total acidity: 6.45 g/L tartaric acid, and volatile acidity: 0.56 g/L acetic acid.

2.3 Human intervention study design

A randomized and controlled 4-week intervention study involving 41 healthy volunteers (33 case and 8 control subjects; 22 women and 19 men; age range 20–65 years), were used in this study[21]. The participants were not suffering from any disease or intestinal disorder, and were not receiving antibiotics or any other medical treatment for at least 6 months before the start of the study or during the study (including the washout period). All the participants were fully informed about the study and gave written informed consent. The study was carried out according to the rules and approval of the Bioethics committee of Hospital "Ramón y Cajal" (Madrid, Spain).

The study was divided into two consecutive periods: a) an initial washout period of 2 weeks (baseline) during which the volunteers did not consume any wine or any other alcoholic beverage and followed a low-polyphenols diet, and b) a period of 4 weeks during which the case volunteers consumed a daily intake of red wine (250 mL) over 28 days (intervention period), divided into two doses. During this latter period, participants also maintained the restrictions for any other alcoholic beverages and followed a low-polyphenols diet. The control group (n=8) followed the same pattern as the case group (n= 33), with the exception that no wine was ingested during this 4-week period.

Each participant provided samples of faeces at two points: a) after the washout period, and b) at the end of the study. In total, 66 samples from the volunteers group (33 before- and 33 after- wine consumption) and 16 samples from the control group (8 before- and 8 after- the 4 weeks study period) were collected. Faeces were immediately frozen and stored at -80 ºC awaiting analysis.

2.4 Preparation of faecal solutions

Faecal samples were thawed at room temperature, homogenized and weighted (1.0 g) in 15 mL sterile conical tubes. 10 mL of sterile saline solution (NaCl 0.9%, Fresenius Kabi, Spain) was added, vortexed and centrifuged (10 min, 10000 rpm, 4 ºC) twice. The supernatant (faecal solution) was filtered by using

0.22 µm pore size polyvinylidene difluoride (PVDF) membrane. Filtered solutions were diluted with water (sample:water, 1:3, v/v) and directly analysed by UHPLC-TOF MS.

2.5 Metabolomic analysis

Metabolomic analysis was performed using a ultra-high performance liquid chromatography (UHPLC) system 1290 from Agilent (Agilent Technologies, Santa Clara, CA, USA) connected to a quadrupole-time-of-flight mass spectrometer (Q/TOF MS) Agilent 6540 equipped with an orthogonal ESI interface (Agilent Jet Stream, AJS) and operating in negative ion mode. The instrument was controlled by a PC running the Mass Hunter Workstation software 4.0 (MH) from Agilent. MS parameters were the following: capillary voltage, 4000 V; nebulizer pressure, 40 psi; drying gas flow rate, 10 L/min; gas temperature, 350 °C; skimmer voltage, 45 V; fragmentor voltage, 110 V. TOF MS accurate mass spectra were recorded across the range of 50-1100 m/z at 1.5 spectra/s in negative ionization mode. Internal mass calibration of the instrument was carried out using an AJS ESI source with an automated calibrant delivery system. The reference compound solution for internal mass calibration of the Q/TOF mass spectrometer containing 9 µM of purine ($[C5H5N4]^-$ at 119.03632 m/z) and 0.5 µM HP-0921, hexakis(1H,1H,3H-tetrafluoropropoxy) phosphazine ($[C18H19O6N3P3F24]^-$ at 921.0031 m/z) in acetonitrile-water (95:5, v/v) was also from Agilent. External calibration of the TOF MS was carried out using a commercial mixture from Agilent with next m/z values: 301.998139, 601.978977, 1033.988109, 1333.968947, 1633.949786, 1933.930624, 2233.911463, 2533.892301, 2833.873139. The sample (2 µL) was injected into an Agilent ZORBAX C8 Rapid Resolution HD column (2.1 × 100 mm, 1.8 µm) maintained at 40 ºC. Elution was performed using 0.01% formic acid in water (solvent A) and 0.01% formic acid in acetonitrile (solvent B), and the following gradient program: 0-30% B in 0-7 min, 30-100%B in 7-11 min and 100% B in 11-14 min. After analysis, the column was re-equilibrated for 7 min

using the initial solvent composition. Each sample was analysed in duplicate. Quality control (QC) samples containing equal volume of all the faecal extracts under study and ABN standards mixture (i.e., a commercial standard mixture containing 42 low- molecular weight compounds of acidic, basic and neutral nature) were injected regularly throughout the run to monitor the performance, stability and reproducibility of the UHPLC-MS method showing a reproducibility of 7.3% and 1.5% inter-day RSD for peaks areas and retention times, respectively.

2.6 Data processing

All raw UHPLC-MS data were extracted and converted to the MS exchange format mzXML using the open-source program Trapper version 4.3.0 (available at http://tools.proteomecenter.org/wiki/index.php?title=Software:trapper). Data processing was performed using MZmine software (version 2.7.2)[22] to obtain a list of peak areas, retention times, and accurate mass to charge ratios (m/z). Settings used in this work are described in Table 1 of Supporting Information. Possible unspecific signals, noise and peaks not common between duplicates of the same sample were removed.

Filtering of the data was then carried out to ensure ions with a high quality: (i) peaks below 3x intensity from the blanks were removed and (ii) peaks not found in at least a 75% of the samples belonging to the same group of volunteers (i.e. before and after wine consumption) or with a high variability within the same group (with a value of median/average > 1.5) were removed. Finally, adducts of the same metabolite were grouped. The resulting output data table of high quality time-aligned detected compounds, with their corresponding retention time, *m/z* and peak area obtained for each sample, was submitted to statistical analysis. Data treatment was carried out using a standard HP computer Z400

(2.8GHz, 4 GB memory) running Windows 7. Further statistical analysis and metabolite identification steps described below were also carried out with the same computer.

2.7 Statistical analysis

The statistical methods used for the data analysis were as follows: the Shapiro Wilk test to verify the normal distribution of the data; t-test for dependent samples and its corresponding nonparametric Wilcoxon matched pairs test to detect metabolites significantly different attributable to wine intake; and principal component analysis (PCA), from correlation matrix, to study variability among samples and metabolites and possible trends in the variables due to wine consumption. A value of $P = 0.05$ was fixed for the level of significance of the tests. The STATISTICA program for Windows, version 7.1 (StatSoft Inc., 1984 2006, www.statsoft.com), was used for data processing.

2.8 Metabolite identification

Assignment of statistically different ($p<0.05$) metabolites was carried out by matching the obtained accurate m/z to those published in the selected databases, namely, KEGG[23], HMDB[24] and Metlin[25] within a mass accuracy window of 10 ppm. Molecular Formula Generator algorithm within MassHunter software (Agilent) was used to support the agreement between the molecular formula generated by the software and the proposed compound from metabolite database search in terms of mass error (ppm) and isotopic pattern similarity. When isomers existed for a given formula, metabolite identification was sorted giving preference to metabolites from central metabolic pathways in KEGG, metabolites already found in faeces and number of databases containing each metabolite. When available, co-injection of standards with faecal samples was performed to confirm the tentatively identified metabolites. An

aliquot of the wine used in the intervention study was also injected in the system to confirm tentative identification of wine compounds detected in the faeces.

3 Results and Discussion

3.1 Metabolic fingerprinting

Firstly, an UHPLC-TOF MS method was developed by using a pooled sample containing equal volumes from all samples under study. Different mobile phase compositions (water with 0.01 or 0.1% HCOOH (v/v) as phase A, and acetonitrile with or without 0.01% HCOOH (v/v) as phase B) and gradient programs were tested in the initial optimization process. The selected chromatographic conditions were those that provided the highest number of peaks with higher resolution and better peak shape. Best peak resolution was achieved in the negative ion mode and using as mobile phase water and acetonitrile, both containing 0.01% HCOOH (v/v). The final selected gradient was as follows: 0-30% B in 0-7 min, 30-100% B in 7-11 min, and 100% B in 11-14 min. A typical UHPLC-TOF MS chromatogram of a faecal solution is shown in Figure 1 of Supporting Information.

Using the developed UHPLC-TOF MS method, metabolic fingerprints of faecal solutions from control group and volunteers before and after wine consumption were obtained after 2µL injection of diluted samples (sample:water, 1:3 v/v). MZmine data processing and the subsequent peak filtering process allowed us to detect 468 high confident metabolites in all the 82 samples. The resulting data matrix with the mean values of peak area from the duplicates was then submitted to statistical analysis.

The overall metabolic differences between volunteer and control groups and differences obtained before and after wine consumption in the volunteers group were first evaluated by PCA. For the control group, no separation was observed between samples before and after wine consumption (data not shown). When wine consumers were considered, faecal samples before and after wine consumption were not

discriminated neither (data not shown). The principal component 1 (PC1) could only explain a 12.5% of the total variance suggesting that although some differences could be found among the groups of volunteers, the vast majority of the metabolome is not altered due to wine consumption as expected in a non-targeted metabolomic approach. Among all samples, it was observed that volunteers 1, 2, 4 and 13 showed higher variability compared to the others. This result was consistent with data of total metabolite content obtained in our previous targeted analysis[21]; volunteers 1 and 13 were classified as high wine-phenolic-metabolizer subjects while volunteers 2 and 4 were classified as moderate metabolizer subjects. After application of the Shapiro Wilk's test that showed no normal distribution of the data, the non-parametric Wilcoxon matched-pairs test was used to evaluate differences in the wine consumer group before and after the wine intake period. From this test, 70 out of the 468 metabolites showed significant differences ($P < 0.05$) between samples before and after wine consumption. Then, these 70 significantly different metabolites were checked in the control group and those found differentially expressed also in the control group (i.e., not associated with wine intake), were removed from the analysis. Finally, we found that the MS response of 37 metabolites was significantly different before and after wine consumption (see Figure 1). Among them, 23 metabolites showed significantly higher MS response after wine consumption, while 14 metabolites showed the opposite behavior as can be seen in Figure 1.

3.2 Identification of compounds related to wine intake

Tentative identifications of the 37 metabolic differences related to moderate consumption of red wine were attempted. After investigation by searching in metabolomics databases and literature, commercial standards, when available, were used to confirm or discard initial metabolite identification. Thus, a total of 21 commercial standards were co-injected with the faecal samples and as a result, L-lysine, ascorbic

acid, pyruvic acid, fumaric acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, γ-valerolactone, 4-methoxyphenylacetic acid, 3-(4-hydroxyphenyl)-propionic acid, 3-(3,4-dimethoxyphenyl)-propionic acid, L-ornithine monohydrochloride, 2-methyl amino benzoic acid and methyl 2-aminobenzoate were discarded as significant metabolites present in the faecal samples. On the other hand, a total of 20 metabolites were tentatively or completely identified whereas the rest of them (17 compounds) remained unknown after this exhaustive investigation (Table 1). Identification was confirmed with commercial standards co-injection in the case of xanthine (ID 4), glutaric acid (ID 9), 3-(3-hydroxyphenyl) propionic acid (ID 15), benzoic acid (ID 17), urobilinogen (ID 19) and stercobilin (ID 20) as shown in Figure 2. Finally, faecal samples spiked with the wine used in the intervention study (1:19, v/v, wine:faecal sample) allowed us to confirm the presence in the faeces of the wine compound tentatively identified as 2-hydroxyglutaric acid (ID 3). Nevertheless, the presence of the other wine compounds could not be discarded since their concentration may be negligible in wine compared to faecal samples.

The tentatively or completely identified metabolites regulated by moderate wine intake were classified into three groups (I, II and III). The group I corresponded to compounds that had been previously reported in wine (wine compounds) (n=6) including 2-hydroxyglutaric acid (ID 3), 2-methylbutyric acid (ID 6), 2,3-pentanedione (ID 10), diethylmalonate (ID 13), 2-phenethyl butyrate (ID 23), and 2-phenylethyl hexanoate (ID 31) (see Table 1). Group II comprised microbial-derived metabolites, i.e., phenolic metabolites derived from the action of gut microbiota on wine polyphenols (n=5), namely 5-(3',4'-dihydroxyphenyl)-gamma-valerolactone (ID 14), 3-(3´-hydroxyphenyl) propionic acid (ID 15), 4-hydroxy-5-(3’-hydroxyphenyl) valeric acid (ID 16), benzoic acid (ID 17) and 4-hydroxy-5-(phenyl) valeric acid (ID 22) (see Table 1). Finally, group III included endogenous metabolites and other compounds derived from non-phenolic nutrient pathways, namely xanthine (ID 4), tricaballylic acid (ID

5), glutaric acid (ID 9) urobilinogen (ID 19), stercobolin (ID 20), docosahexaenoic acid methyl ester (ID 26), deoxycholic acid (ID 27), sulfolithocholic acid (ID 28) and cholesterol sulfate (ID 37).

3.3 Metabolic interpretation

Identification (tentative or complete) of a total of 20 faecal metabolites out of the 37 metabolites that showed significant differences ($p<0.05$) before and after wine consumption was carried out (Figure 1, Table 1). Some of these identified compounds corresponded to wine compounds (named as group I) as their presence was confirmed in the proper wine used in the study (i.e., 2-hydroxyglutaric acid, ID 3) or already reported in the literature: ID 3[27,28], 2-methylbutyric acid (ID 6)[29], 2,3-pentanedione (ID 10)[30-32], diethylmalonate (ID 13)33, 2-phenethyl butyrate (ID 23)[34], and 2-phenylethyl hexanoate (ID 31)[35] (Table 1). Among them, 2-methylbutyric acid (ID 6) has also been reported in faeces[36]. As expected, the content of all wine compounds in faeces significantly increased after wine intake (Figure 1).

Among the compounds classified as microbial-derived metabolites, the content of 5-(3',4'-dihydroxyphenyl)-γ valerolactone (ID 14), 4-hydroxy-5-(3'-hydroxyphenyl) valeric acid (ID 16), benzoic acid (ID 17) and 4-hydroxy-5-(phenyl) valeric acid (ID 22) in faeces increased after wine intake. This is in line with our previous targeted analysis of these same samples[21]. Phenyl-γ-valerolactones and 4-hydroxy-5-hydroxyphenyl valeric acid derivatives are characteristic metabolites exclusively derived from the catabolism of flavan-3-ols[4] and, as expected, were up-regulated by wine intake. As well as benzoic acid, which is amongst the end-products of the microbial catabolism of flavan-3-ols and it was also quantified in the wine used in this intervention study[21]. On the contrary, and in agreement with previous targeted studies[21,37], we found a decrease in the content of 3-(3-hydroxyphenyl) propionic acid, a microbial metabolite also derived from the catabolism of flavan-3-ols,

after wine polyphenols intake. This fact may be due to a high dehydroxylation activity of the intestinal microbiota resulting in phenylpropionic acid, as previously reported with grape seed polyphenols[38].

Finally, other metabolites related to the moderate consumption of red wine corresponding to endogenous metabolites and/or other compounds derived from non-phenolic nutrient pathways were found (named as group III). Among them, xanthine (ID 4) (that was confirmed co-injecting the standard) is a purine base and an intermediate in the degradation of adenosine monophosphate to uric acid, being formed mainly by oxidation of hypoxanthine by xanthine oxidase (XO) producing hydrogen peroxide or a superoxide radical. We found lower concentration of xanthine in faeces after wine intake, which is in accordance with previous studies that suggest that the antioxidant properties assigned to bioactive compounds such as flavonoids[39] and resveratrol[40,41] is due to their inhibitory effect on the activity of XO.

Glutaric acid (ID 9) has been previously reported on faeces[36] and its identification was also confirmed injecting its standard. It is a dicarboxylic acid produced during the metabolism of some amino acids, including lysine and tryptophan. Moreover, it can be produced by anaerobic fermentative gut bacteria (together with lactic acid)[42]. In the present study we found higher content of glutaric acid in faeces after wine intake.

Urobilinogen (ID 19) and stercobilin (ID 20) –that were confirmed with their commercial standards- are byproducts of bilirubin degradation. Urobilinogen is formed in the intestines by bacterial action, especially *Clostridium* spp.[43]. Part is reabsorbed, taken up by the hepatocytes into the circulation and excreted by the kidney. The urobilinogen remaining in the intestine (stercobilinogen) is oxidized to brown stercobilin which gives the faeces their characteristic color. These metabolites were found down-regulated by wine consumption which is in line with numerous studies that indicate that flavan-3-ol-rich

sources, such as wine, may modulate the intestinal microbiota increasing beneficial bacteria but inhibiting other groups such as *Clostridium* spp.[44-49].

Although it was tentatively confirmed, tricarballylic acid (ID 5) is an endogenous metabolite whose presence has also been reported in faeces[36]. The content of this metabolite decreased after wine consumption.

Besides, wine consumption led to a higher content of docosahexaenoic acid methyl ester (ID 26) in faeces, although identification was tentative. The corresponding docosahexaenoic acid (DHA) is an omega-3 essential fatty acid associated with numerous potential health effects. Using *in vitro* and *ex vivo* approaches to investigate the effects of red wine polyphenols on the oxidizability of the major fatty acids of human plasma, Cazzola et al.[50] suggested that wine polyphenols protect (n-3) polyunsaturated fatty acids such as DHA from oxidation in plasma. Apart from this direct effect of wine polyphenols in preserving fatty acids (i.e., DHA), favourable interactions between flavan-3-ols and gut microbiota might promote growth of beneficial intestinal bacteria groups such as *Lactobacillus*[49], species that have been suggested to regulate polyunsaturated fatty acids absorption[51]. This is in agreement with the increase of DHA observed in feaces after wine consumption (Figure 1).

Gut bacterial enzymes are also involved in the metabolism of cholesterol and its metabolites bile acids. In the present study we found that the secondary bile acids bile acids deoxycholic acid (ID 27) and sulfolithocholic acid (ID 28), and cholesterol sulfate (ID 37) may be regulated by wine intake. Although it is known that dietary intake of phenolic-rich sources affect their metabolism, different effects have been observed. Yunghan et al.[52] demonstrated the suppressing effect of some polyphenols (including curcumin, caffeic acid, catechin, rutin, and ellagic acid) on faecal levels of deoxycholic acid and lithocholic acid in rats fed with a high-fat diet by suppressing the biosynthesis of bile acids from cholesterol together with cholesterol biosynthesis. Moreover, the transformation of primary bile acids to

secondary bile acids in the intestines is mainly performed by anaerobic bacteria, including the genera *Clostridium*[53], inhibited by flavan-3-ol-rich sources[49]. In line with this, we observed a decrease in the content of sulfolithocholic acid in faeces after wine intake. In contrast, we found the content of deoxycholic acid up-regulated by wine. The same effect was observed in a work developed by Montilla et al.[54] when red wine was simultaneously supplemented to high-cholesterol diet in hypercholesterolemic rats. The authors observed that the excretion of faecal bile acids, as well as their plasma and hepatic concentrations were increased significantly, which indicated an increase in the cholesterol degradation. Finally, Shimizu-Ibuka et al.[55] studied the hypocholesterolemic effect of peanut skin in rats and found that it had a strong effect on reducing the serum cholesterol level and little effect on the hepatic cholesterol level, increasing the total amount of cholesterol in the faeces. This study suggested that polyphenols included in the extract would have had an anti-hypercholesterolemic effect by inhibiting the absorption of exogenous cholesterol and lipids in the small intestine. In our study, we observed an increase in the faecal content of cholesterol sulfate after wine intake.

4 Conclusions

A non-targeted screening of new metabolic markers in faeces is challenging for any metabolomic study. The main reason is, leaving aside the impact of diet and habits, the high variability and diversity of faeces compounds, which can be explained as faecal metabolome is affected by co-metabolism and metabolic interactions between gut bacteria and human intestine cells[26]. In this paper, a protocol for MS-based metabolite fingerprinting of faecal metabolome has been successfully developed. A high-reproducible and sensitive LC-MS method has been developed for the analysis of faecal metabolome and has been successfully applied for the discrimination of volunteers after moderate wine consumption. It is noteworthy that 37 biomarkers related to wine consumption have been unravelled in this work

overcoming the high intra- and inter- individual inherent variability of faecal metabolite content. As far as we know, this is the first study of the metabolomic fingerprint of faecal samples after the consumption of wine. Our results confirm intact passage of certain wine compounds (i.e., ID3, tentatively identified as 2-hydroxyglutaric acid) through the gastrointestinal track. Also, metabolites derived from the action of gut microbiota in wine polyphenols (i.e., flavan-3-ols) seemed to profoundly impact on the global faecal metabolite profile, as expected from previous phenolic-targeted studies. Besides, wine consumption also impacts on other nutrient pathways (i.e., lipids) and endogenous metabolites (i.e., xanthine and bilirubin). Overall, the differences observed in the fecal metabolome after wine consumption reflect changes in microbiota composition and functionality, that could in turn be partly attributed to modulation effects by wine polyphenols and/or their metabolites. Further metagenomic approaches could clarify this point.

5 References

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

FIGURE CAPTIONS

Figure 1. Bar plot representation of median values for all significantly increased (A) and decreased (B) metabolites after wine consumption. Numbers refer to the metabolite identification in Table 1.

Figure 2. Extracted ion chromatograms of a faecal sample (black line), faecal sample spiked with 2.5 µg/mL 3-(3-hydroxyphenyl) propionic acid (ID 15), 1 µg/mL glutaric acid (ID 9), 1 µg/mL benzoic acid (ID 17), 1 µg/mL xanthine (ID 4), 3.3 µg/mL stercobilin (ID 20) and 1.2 mg/mL urobilinogen (ID 19) (blue line) and commercial standard (red line). UHPLC-MS conditions as described in materials and methods.

Table 1. Tentative identification of potential metabolic biomarkers in fecal samples after moderate wine consumption.

Metabolite ID	RT (min)	Detected m/z	Assigned Ion	Formula	Error (ppm)	Effect of wine intake	Identification	
							Tentative	Confirmed[a]
1	0.60	**256.059**	-	-	-	down-regulated	Unknown	
2	0.64	**160.842**	-	-	-	up-regulated	Unknown	
3	0.96	**147.030**	$[M-H]^-$	$C_5H_8O_5$	-1.3	up-regulated	2- Hydroxyglutaric acid[b]	
4	1.18	**151.026**	$[M-H]^-$	$C_5H_4N_4O_2$	0.4	down-regulated		Xanthine
5	1.43	**175.025**	$[M-H]^-$	$C_6H_8O_6$	1.2	down-regulated	Tricarballylic acid	
6	2.12	**147.065**	$[M+FA-H]^-$	$C_5H_{10}O_2$	9.7	up-regulated	2-Methylbutyric acid	
7	2.13	**392.121**	-	-	-	down-regulated	Unknown	
8	2.15	**189.040**	$[M+FA-H]^-$	$C_6H_8O_4$	2.4	up-regulated	Unknown	
9	2.16	**131.035**	$[M-H]^-$	$C_5H_8O_4$	0.4	up-regulated		Glutaric acid
10	2.93	**145.051**	$[M+FA-H]^-$	$C_5H_8O_2$	0.7	up-regulated	2,3-Pentanedione	
11	3.22	**150.056**	$[M-H]^-$	$C_8H_9NO_2$	1.0	up-regulated	Unknown	
12	3.38	**141.050**	-	-	-	down-regulated	Unknown	
13	3.91	**159.066**	$[M-H]^-$	$C_7H_{12}O_4$	1.3	up-regulated	Diethylmalonate	
14	4.61	**207.067**	$[M-H]^-$	$C_{11}H_{12}O_4$	-4.5	up-regulated	5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone	
15	4.93	**165.055**	$[M-H]^-$	$C_9H_{10}O_3$	2.1	down-regulated		3-(3-Hydroxyphenyl)propionic acid
16	5.32	**209.081**	$[M-H]^-$	$C_{11}H_{14}O_4$	2.9	up-regulated	4-Hydroxy-5-(3'-hydroxyphenyl)- valeric acid	
17	6.03	**121.030**	$[M-H]^-$	$C_7H_6O_2$	-1.7	up-regulated		Benzoic acid
18	6.40	**467.231**	-	-	-	down-regulated	Unknown	
19	7.16	**591.318**	$[M-H]^-$	$C_{33}H_{44}N_4O_6$	0.9	down-regulated		Urobilinogen
20	7.21	**593.334**	$[M-H]^-$	$C_{33}H_{46}N_4O_6$	1.5	down-regulated		Stercobilin
21	7.33	**319.166**	-	-	-	down-regulated	Unknown	
22	7.48	**193.087**	$[M-H]^-$	$C_{11}H_{14}O_3$	2.3	up-regulated	4-Hydroxy-5-(phenyl)-valeric acid	
23	7.87	**191.107**	$[M-H]^-$	$C_{12}H_{16}O_2$	4.6	up-regulated	2-Phenethyl butyrate	

24	8.06	**319.190**	-	-	-	up-regulated	Unknown	
25	8.75	**303.131**	-	-	-	up-regulated	Unknown	
26	9.55	**387.254**	$[M+FA-H]^-$	$C_{23}H_{34}O_2$	-0.5	up-regulated	Docosahexaenoic acid methyl ester	
27	9.78	**391.285**	$[M-H]^-$	$C_{24}H_{40}O_4$	1.1	up-regulated	Deoxycholic acid	
28	11.50	**455.247**	$[M-H]^-$	$C_{24}H_{40}O_6S$	-0.2	down-regulated	Sulfolithocholic acid	
29	11.66	**366.941**	-	-	-	up-regulated	Unknown	
30	11.70	**982.992**	-	-	-	down-regulated	Unknown	
31	11.83	**265.144**	$[M+FA-H]^-$	$C_{14}H_{20}O_2$	0.7	up-regulated	2-Phenylethyl hexanoate	
32	11.92	**279.165**	-	-	-	down-regulated	Unknown	
33	11.94	**353.200**	-	-	-	down-regulated	Unknown	
34	12.02	**532.928**	-	-	-	up-regulated	Unknown	
35	12.25	**648.916**	-	-	-	up-regulated	Unknown	
36	12.33	**698.914**	-	-	-	up-regulated	Unknown	
37	13.65	**465.306**	$[M-H]^-$	$C_{27}H_{46}O_4S$	-2.5	up-regulated	Cholesterol sulfate	

a) Metabolite identification confirmed with commercial standards; b) Confirmed in the wine used in the study

5.2. Efecto del consumo moderado de vino tinto sobre la función intestinal

En el intestino humano tienen lugar numerosos procesos fisiológicos cruciales para el mantenimiento de la salud humana entre los que destacan la digestión y absorción de nutrientes, y funciones de defensa frente a antígenos/patógenos que acceden al sistema digestivo a través de los alimentos, agua y aire. Muchas de estas funciones se deben o están influenciadas, en gran parte, por la presencia de microorganismos en el intestino (Guarner y Magelada, 2003). La microbiota intestinal posee un enorme potencial enzimático que le permite desempeñar una amplia variedad de funciones metabólicas, además de participar en los sistemas de defensa y mantenimiento de la estructura intestinal.

Estudios recientes indican que el consumo moderado de vino parece modificar la proporción relativa de algunos grupos bacterianos a nivel intestinal (Queipo-Ortuño y col., 2012). Por otro lado, al igual que se ha sugerido para otros tipos de polifenoles como las isoflavonas (Bolca y col., 2009) o las urolitinas (Tomas-Barberan y col., 2014), la microbiota intestinal podría condicionar la capacidad de los individuos para metabolizar los polifenoles del vino, y por ende, la susceptibilidad de los mismos a los beneficios saludables atribuidos al consumo moderado de vino. Determinar los cambios en la composición de la microbiota fecal como consecuencia del consumo moderado de vino es un primer paso para evaluar sus posibles efectos beneficiosos sobre la función intestinal, así como para establecer una posible relación entre la microbiota y la capacidad para metabolizar los polifenoles del vino por parte de los seres humanos. Las nuevas técnicas de secuenciación masiva como la pirosecuenciación del gen 16S rRNA (Metagenómica) están actualmente en auge y su utilización permitiría un análisis completo de las poblaciones bacterianas intestinales en muestras fecales y abrirá nuevas perspectivas para comprender el papel de la microbiota en la función y salud intestinal. Sin embargo, los estudios metagenómicos sobre la relación entre la microbiota intestinal y la capacidad del organismo humano de metabolizar los alimentos y sus componentes son muy escasos, y en el caso concreto del vino, estos estudios no han sido todavía abordados.

Algunos autores han sugerido que el vino y, concretamente, los polifenoles del vino, podrían ejercer un efecto sobre la respuesta inmune y/o inflamatoria intestinal, como han revelado varios estudios realizados con modelos celulares (Biasi y col., 2013; Nunes y col., 2013), los cuales han

encontrado cambios en la concentración de algunas citoquinas de gran relevancia en el desarrollo de la respuesta inflamatoria intestinal.

Como continuación del objetivo 1 y con la finalidad de evaluar los cambios en el metaboloma fecal y otros factores relacionados con la función intestinal en heces humanas tras el consumo moderado de vino en voluntarios sanos, en este apartado se recogen los resultados relativos a la composición de la microbiota fecal y de marcadores inmunológicos.

Por tanto, este apartado responde a los siguientes sub-objetivos:

1. Evaluar el efecto del consumo moderado de vino sobre las poblaciones microbianas del intestino mediante el análisis metagenómico de heces procedentes de voluntarios participantes en un estudio de intervención nutricional con vino (**Publicación 5.2.1).**

2. Evaluar el efecto del consumo moderado de vino sobre la función inmune intestinal mediante el análisis inmuno-enzimático de marcadores de inflamación (inmunoglobulinas y citoquinas) en las heces procedentes de voluntarios participantes en un estudio de intervención nutricional con vino (**Publicación 5.2.2**).

A continuación, se presentan los resultados de estos trabajos en forma de publicaciones científicas.

<u>Publicación 5.2.1</u> Análisis metagenómico de las poblaciones bacterianas en muestras fecales de sujetos en un estudio de intervención con vino tinto (MANUSCRITO EN PREPARACIÓN)

Evaluation of the Changes in the Human Faecal Bacterial Populations after Moderate Consumption of Red Wine by a Metagenomic Approach (In preparation)

Resumen:

This work aims to ascertain the effects of moderate consumption of red wine in the intestinal bacteria populations. Faecal samples from an intervention study that consisted in a moderate and regular consumption of red wine by healthy volunteers were analysed by a metagenomic analysis using a next-generation sequencer of 16S rRNA. The results showed no significant changes in the bacterial populations after wine consumption. However, a deeper analysis of the results showed that, according to a previous classification of the volunteers (Muñoz-González y col., 2013) into high, moderate and low metabolizers, the bacterial composition was different in those volunteers classified as high metabolizers. In this group, a higher proportion of Actinobacteria, edge level, *Bifidobacterium*, a genus level, and *Bifidobacterium ruminantium* and *Bifidobacterium sp. 113*, at the species level were found.

Abstract

This work aims to ascertain the effects of moderate consumption of red wine in the bacterial populations of faecal samples from an intervention study that consisted in a moderate and regular consumption of red wine by healthy volunteers. DNA from samples was extracted and a metagenomic analysis using 16 S RNA sequencing in Ilumina Platform. The results showed no significative changes after wine consumption of red wine. However, a deeper analysis of the results showed that, according to a previous classification of the volunteers (Muñoz-González y col., 2013) into high, moderate and low metabolizers, the bacterial composition was different in those volunteers classified as high metabolizers. In this group, Actinobacteria filo was significantly higher in comparison with the moderate and low metabolizers. At the genus level, *Bifidobacterium* genus was also significantly higher and, at specie level, *Bifidobacterium* and *Bifidobactrium* were...Firmicutes filo was slightly lower and 6 species were lower in the high metabolizers group.

Materials and methods

Human intervention study with wine

A randomized, crossover, controlled intervention study that consisted in the moderate consumption of a young Pinot Noir red wine with a high phenolic content (1758 mg Gallic acid equivalents / L) by healthy human subjects (n=43, 34 cases and 9 controls) during four weeks was performed. The study consisted of two periods, a washout period (2 weeks) in which restricted consumption of foods rich in polyphenols, and other intervention period (4 weeks) with wine. The 9 control subjects followed the same guidelines as volunteers, with the exception that no wine ingested during the 28 days of intervention. The study was carried out according to the rules and approval of the Bioethics committee.

DNA extraction from fecal samples

Fecal samples were collected after the washout period and the intervention period and stored at -80 ºC awaiting analysis. For preparation of fecal waters, samples were thawed at room temperature, weighted (0.1 g) in a microcentrifuge tube, and suspended in 0.5 mL of extraction buffer (200 mM Tris–HCl pH 7.5, 0.5% SDS, 25 mM EDTA, 250 mM NaCl, 20 mg/mL lysozyme, 5 µg/mL lysostaphin) and 0.3 mL of 3 M Na acetate. Then, mechanical lysis was performed by three times of bead-beating with 0.1 mm diameter zirconia/silica beads (Sigma) using a FastPrep disruptor (QBioGene, Irvine, CA, USA) at a speed setting of 6.0 m/s for 30 s. The lysate solution was treated with 0.1 mg/mL of proteinase K (Sigma), and incubated for 30 min at 37°C. Following incubation, 0.1 mL of 1.5 M NaCl was added to the lysate and mixed. After incubation for 5 min at room temperature, the mixture was centrifuged at 16,000 ×g to pellet the insoluble cell debris. The supernatant was transferred into a new tube and extracted twice with an equal volume of phenol/chloroform/isoamyl-alcohol (25:24:1) (Sigma). The DNA was precipitated by adding 0.6 volumes of isopropanol (Sigma) and incubating at -20°C for 1 h. The DNA was pelleted, washed with 70% ethanol, allowed to air dry, and finally resuspended in TE buffer. The QIAEX II Gel Extraction Kit was used to purified and concentrate DNA. The DNA yield was measured using a NanoDrop® ND-1000 UV spectrophotometer (Nano-Drop Technologies, Wilmington, DE).

16S rRNA gene Illumina MiSeq sequencing

The DNA samples were amplified by PCR using primers 27F-DegL (5’-GTTYGATYMTGGCTCAG-3’) in combination with an equimolar mixture of two reverse primers, 338R-I (5’-GCWGCCTCCCGTAGGAGT-3’) and 338R-II (5’GCWGCCACCCGTAGGTGT-3’) generating an approximately 345 bp amplicons from the V1 to V2 hypervariable regions of 16S rDNA genes. PCR

amplification was performed using the Fast Start High Fidelity PCR System dNTP Pack (Roche, Mannheim, Germany)in a total volume of 25 μL containing 2.5 μL 10× Reaction Buffer and 1.25 units of Fast Start High Fidelity Enzyme Blend (Roche), 0.4 μM of each primer, and 10 ng template DNA. Thermal cycling conditions were as follows: an initial denaturation at 95°C for 2 min, and 35 cycles at 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. Following amplification, 2 μl of PCR product was used for agarose gel (1%) detection.

Barcodes used for Illumina sequencing were appended to 3' and 5' terminal ends of PCR amplicons in a second PCR to allow separation of forward and reverse sequences. All primers were synthesized by Isogen Life Sciences (Castelldefels, Spain).

Barcoded PCR products from all samples were pooled at approximately equal molar DNA concentrations and run on a preparative agarose gel. The correct sized band was excised and purified using a QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany) and quantified with PicoGreen (BMG Labtech, Jena, Germany). The 16S DNA amplicons were sequenced on an Illumina MiSeq (Illumina Inc., San Diego, CA, USA) technology reads 2x250.

Statistical analysis

Wilcoxon non-parametric test was used to test the effect of wine moderate consumption on the bacterial populations in the fecal samples. One way Analysis of Variance (ANOVA) and LSD test was used to ascertain the differences on the fecal bacterial populations between the high, moderate and low metabolizers groups.

Results

Table 1. Relative percentages, edge level, determined in the fecal samples of Control, High metabolizers, Moderate metabolizers and Low metabolizers groups, before and after consumption.

	CONTROL		WINE CONSUMERS					
			Low metabolizers		Moderate metabolizers		High metabolizers	
	Before	After	Before	After	Before	After	Before	After
Firmicutes	80.3±7.2	81.3±8.2	80.3±8.8b	77.4±9.5	82.3±8.4b	78.7±9.7	68.7±8.8a *(p=0.058)*	69.3±14.7
Actinobacteria	15.2±10.3	13.0±11.0	15.3±8.2a	18.0±8.5	11.6±7.7a	13.7±9.9	27.1±10.4b *(p=0.043)*	24.3±16.2
Bacteroidetes	3.92±3.38	5.21±7.54	3.85±4.88a	2.92±2.28	4.23±6.78a	4.95±9.07	2.90±2.11a	4.79±1.93
Proteobacteria	0.367±0.445	0.406±0.448	0.482±0.407a	1.58±1.23	1.71±2.76a	2.51±2.76	1.10±1.08a	1.40±1.42
Verrucomicrobia	0.137±0.120	0.0562±0.0961	0.0509±0.0889a	0.0155±0.0256	0.0306±0.0302a	0.0273±0.0378	0.0492±0.0501a	0.0664±0.0823
Tenericutes	0.0368±0.0453	0.0158±0.0221	0.00893±0.01680a	0.00986±0.01570	0.00461±0.00602a	0.0231±0.0287* *(p=0.043)*	0.104±0.155a	0.0972±0.1247
Fusobacteria	0.0382±0.0665	0.0311±0.0437	0.0225±0.0447a	0.0075±0.0057	0.0333±0.0586a	0.0311±0.0655	0.0178±0.0304a	0.0199±0.0243
Cyanobacteria	0.00476±0.00435	0.0106±0.0152	0.00467±0.00259a	0.105±0.179	0.00828±0.00973a	0.00446±0.00197	0.0119±0.0180a	0.0187±0.0292
Others	0.0328±0.0603	0.0510±0.1050	0.0071±0.0072a	0.0146±0.0199	0.0136±0.0107a	0.00991±0.00682	0.0392±0.0765a	0.0040±0.0009

a-b: Different letters mean significant differences, before wine intake, between gro

Table 2. Relative percentages, genus level, determined in the fecal samples of Control, High metabolizers, Moderate metabolizers and Low metabolizers groups, before and after consumption.

	CONTROL		WINE CONSUMERS					
			LOW METABOLIZERS		MODERATE METABOLIZERS		HIGH METABOLIZERS	
	Before	After	Before	After	Before	After	Before	After
Bifidobacterium	8.40±6.04	7.29±7.33	10.5±7.7a	13.0±7.7	8.02±6.96a	9.20±9.66	23.4±10.6b *(p=0.032)*	20.6±17.2
Clostridium	6.38±2.50	7.97±6.38	9.00±5.12a	5.75±2.45	7.61±5.83a	10.6±8.4	4.61±2.02a	3.59±1.96
Ruminococcus	7.66±1.27	6.27±3.06	5.26±3.56a	5.12±3.07	6.29±4.37a	7.66±6.25	4.20±1.36a	5.26±2.71
Eubacterium	4.29±2.94	4.77±4.55	3.26±2.66a	2.85±1.73	5.19±4.58a	5.95±4.22	1.76±1.62a	3.33±3.57
Collinsella	5.46±4.84	4.60±4.12	3.03±3.05a	3.05±2.82	2.46±1.37a	3.23±2.42	2.06±1.89a	2.29±2.10
Catenibacterium	4.06±9.07	7.76±17.33	1.02±2.05a	0.748±1.671	1.11±2.49a	0.718±1.600	4.10±9.17a	3.19±7.06
Streptococcus	0.674±0.429	0.710±0.531	1.76±1.77a	5.39±5.21	3.21±5.89a	0.964±1.014	4.38±8.10a	0.524±0.526
Sporacetigenium	1.06±0.60	1.08±0.90	1.12±0.43a	1.19±0.97	0.809±0.523a	0.971±0.529	0.916±0.679a	0.789±0.522
Enterococcus	0.0268±0.0581	0.0613±0.1244	0.751±1.09a	0.226±0.319	0.483±1.069a	0.00595±0.00820	0.394±0.734a	0.244±0.451
Roseburia	0.563±0.357	0.681±0.175	0.880±0.510a	0.932±0.397	1.01±0.57a	0.839±0.338	0.880±0.617a	1.11±0.76
Dorea	0.745±0.385	0.765±0.459	0.771±0.210a	0.986±0.697	0.991±0.411a	1.13±0.65	0.762±0.504a	1.33±0.55
Faecalibacterium	1.23±1.23	1.05±1.00	0.679±0.587a	0.950±0.735	0.218±0.198a	0.182±0.194	0.841±0.625a	0.897±0.951
Bacteroides	0.523±0.692	1.52±2.78	1.07±1.83a	1.00±1.49	1.04±1.71a	1.26±2.50	0.191±0.081a	0.499±0.617
Anaerostipes	0.585±0.475	0.722±0.575	0.780±0.419a	1.01±0.41	0.686±0.573a	0.832±0.640	0.740±0.477a	0.448±0.437
Dialister	0.0302±0.0666	0.0439±0.0970	0.141±0.313a	0.000646±0.001191	0.0990±0.2213a	0.0871±0.0871	0.000342±0.000764a	0.000117±0.000261
Turicibacter	0.232±0.446	0.685±1.422	0.889±1.405a	0.255±0.318	0.232±0.198a	0.423±0.812	0.131±0.198a	0.0210±0.0369
Escherichia	0.0987±0.0473	0.0763±0.0762	0.137±0.087a	0.123±0.094	0.105±0.121a	0.113±0.098	0.0467±0.0271a	0.0504±0.0288
Blautia	0.278±0.199	0.243±0.148	0.280±0.085a	0.172±0.091* *(p=0.049)*	0.191±0.194a	0.185±0.124	0.237±0.150a	0.266±0.134
Lactococcus	0.0164±0.0362	0.0123±0.0271	0.00223±0.00193a	0.0587±0.0856	0.211±0.462a	0.0739±0.0739	0.00658±0.01439a	0.000450±0.000451
Coprococcus	0.137±0.063	0.119±0.068	0.208±0.166a	0.321±0.271	0.157±0.182a	0.0558±0.0522	0.122±0.086a	0.196±0.151
Adlercreutzia	0.0818±0.0773	0.0614±0.0651	0.0434±0.0471a	0.0787±0.1020	0.0176±0.0233a	0.0261±0.0347	0.0179±0.0207a	0.0347±0.0265
Eggerthella	0.138±0.217	0.101±0.091	0.149±0.270a	0.180±0.344	0.162±0.317a	0.0756±0.1176	0.0454±0.0634a	0.166±0.259
Actinomyces	0.0987±0.0473	0.0763±0.076	0.137±0.087a	0.123±0.094	0.105±0.121a	0.113±0.098	0.0467±0.0271a	0.0504±0.0288

		2						
Leifsonia	0.000290±0.000266	0.158±0.353	0.0618±0.1290a	0.0693±0.1505	0.162±0.276a	0.0656±0.1461	0.0292±0.0653a	0.0686±0.1532
Lactobacillus	0.123±0.136	0.0601±0.0680	0.0818±0.0679a	0.281±0.351	0.0918±0.1059a	0.0404±0.0381	0.00535±0.00455a	0.475±0.963
Peptococcus	0.0483±0.0908	0.0523±0.0948	0.00934±0.01297a	0.0288±0.0418	0.0527±0.0726a	0.0301±0.0490	0.00393±0.00711a	0.00314±0.00566
Bosea	0.0000873±0.0001951	0.0263±0.0585	0.0405±0.0570a	0.312±0.695	0.212±0.386a	0.0190±0.0421	0.165±0.369a	0.0138±0.0289
Shigella	0.0202±0.0411	0.685±1.150	0.0786±0.1411a	0.916±1.774	3.59±7.78a	0.176±0.368	0.0123±0.0176a	0.00924±0.01069
Parabacteroides	0.0262±0.0426	0.0495±0.0842	0.0375±0.0587a	0.0567±0.1005	0.114±0.226a	0.0323±0.0131	0.0246±0.0344a	0.0502±0.0492
Proteus	0.0814±0.1372	0.0308±0.0473	0.0642±0.1094a	0.0258±0.0501* *(p=0.021)*	0.0107±0.0155a	0.0956±0.1988	0.0213±0.0165a	0.467±1.024
Others	0.945±0.345	0.746±0.546	1.18±0.82a	1.23±1.04	0.690±0.514a	0.522±0.226	1.10±0.77a	0.786±0.290

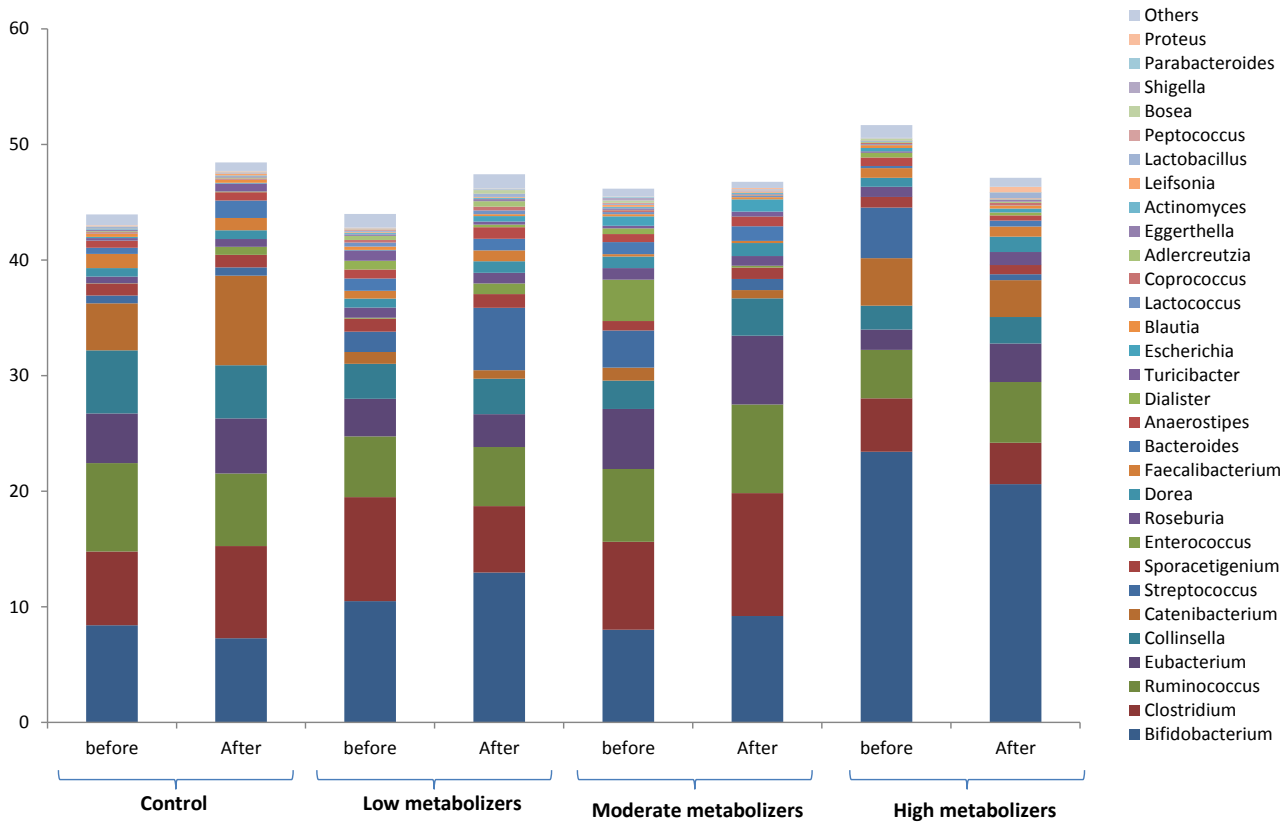
a-b: Different letters mean significant differences, before wine intake, between groups

Table 3. Relative percentages , specie level, determined in the fecal samples of Control, High metabolizers, Moderate metabolizers and Low metabolizers groups, before consumption.

	WINE CONSUMERS		
	Low metabolizers	**Moderate metabolizers**	**High metabolizers**
Uncultured Spartobacteria bacterium	0.0003±0.0003b	0.0000±0.0000a	0.0000±0.0000a
Clostridiales bacterium 10-3b	0.0059±0.0042b	0.0000±0.0000a	0.0012±0.0016a
Streptococcus parasanguinis	0.0464±0.0243b	0.0148±0.0283a	0.0014±0.0013a
Ruminococcus sp. CO27	0.0080±0.0062b	0.0012±0.000a	0.0022±0.0015a
Staphylococcus aureus	0.0035±0.0034b	0.0000±0.0000a	0.0000±0.0000a
Escherichia sp. SW86	0.0000±0.0000a	0.0003±0.0003b	0.0000±0.0000a
Bifidobacterium ruminantium	0.0056±0.0075a	0.0017±0.0015a	0.0596±0.0546b
Uncultured Clostridales bacterium	0.0238±0.0273a	0.1523±0.1163b	0.0360±0.0477a
Bifidobacterium sp. 113	0.0089±0.0099a	0.0034±0.0041a	0.0445±0.0346b
Uncultured Staphylococcus sp.	0.0079±0.0049b	0.0021±0.0031a	0.0002±0.0002a
Uncultured Clostridales Family XIII bacterium	0.0003±0.0004b	0.0010±0.0008b	0.0000±0.0000a
Streptococcus sp. oral clone BP2-57	0.0000±0.0000a	0.0001±0.0001a	0.0008±0.0007b
Uncultured Dechloromonas sp.	0.0000±0.0000a	0.0006±0.0004b	0.0001±0.0003a
Uncultured Comamonas sp.	0.0000±0.0000a	0.0000±0.0000a	0.0004±0.0004b
Clostridium sp. enrichment culture clone VN_TX2-19	0.0004±0.0005b	0.0000±0.0000a	0.0000±0.0000a
Collinsella tanakaei	0.0000±0.0000a	0.1190±0.0889b	0.0155±0.0346a
Actinomyces naeslundii	0.0010±0.0005b	0.0000±0.0000a	0.0008±0.0009b
Actinobaculum sp. oral taxon 183	0.0004±0.0005a	0.0014±0.0011b	0.0002±0.0003a
Actinomyces sp. oral clone CT068	0.0011±0.0007b	0.0001±0.0003a	0.0005±0.0006ab

a-b: Different letters mean significant differences, before wine intake, between groups.

Figure 1. Relative abundance of the most abundant genus.



<u>Publicación 5.2.2</u> El consumo moderado de vino puede modular la respuesta inflamatoria intestinal

Moderate Consumption of Red Wine Can Modulate Human Intestinal Inflammatory Response

Irene Muñoz-González, Irene Espinosa-Martos, Juan M. Rodríguez, Pedro J. Martín-Álvarez, Begoña Bartolomé, M. Victoria Moreno-Arribas. *Journal of Agricultural and Food Chemistry*, **2014** (Enviado para su publicación)

Resumen:

Este trabajo tiene como objetivo evaluar el efecto potencial del consumo moderado de vino tinto sobre la respuesta inflamatoria intestinal, mediante la medida de marcadores inmunes en heces humanas. Para ello, se analizaron 24 marcadores inmunológicos en las heces procedentes de un estudio de intervención con voluntarios sanos (n=34), antes y después del consumo de vino tinto rico en polifenoles durante 4 semanas. Una exploración exhaustiva de los valores de marcadores inmunes antes del consumo de vino, permitió diferenciar un subgrupo de 6 voluntarios que mostraron valores inusualmente altos de citoquinas. Para este grupo, el análisis estadístico reveló diferencias significativas en el contenido de 16 de los 24 marcadores, reduciendo éstos hasta valores normales. Dicha disminución fue especialmente notable en el caso de las citoquinas que promueven el inicio del proceso inflamatorio, TNF-α, IL-6 e IFN-γ. Por lo tanto, este estudio muestra, por primera vez, que el consumo moderado de vino tinto podría modular la respuesta inflamatoria intestinal *in vivo,* al menos determinada como el nivel de marcadores inmunes en heces.

ABSTRACT

This work aims to ascertain the effects of moderate consumption of wine in the intestinal inflammation response as measured in human feces. For that purpose, 24 immune markers were analysed in feces from healthy volunteers (n = 34) before and after consumption of red wine for 4 weeks. A detailed exploration of the values before wine consumption differentiated a 6-volunteer subgroup showing unusually high values of cytokines. Interestingly, for this subgroup, consumption of wine significantly ($P < 0.05$) reduced the content of 16 out of 24 markers down to usual values, especially noticeable for those cytokines that promote initial inflammation (TNF-α, IL-6 and IFN-γ). On the contrary, no significant differences in the concentration of any immune markers were observed before and after wine consumption for the rest of the volunteers (n=28), exhibiting low cytokine values before wine consumption. Additionally, significant and negative correlations among the cytokines IFN-ϒ, IL-8 and IL-6 and the total fecal content of phenolic metabolites were found for the high-cytokine-values subgroup, before wine intake. Therefore, this study shows, for the first time, that moderate consumption of red wine could modulate inflammatory intestinal response *in vivo*.

Keywords: red wine, immune markers, cytokines, feces

ABBREVIATIONS: **G-CSF**, granulocyte colony stimulating factor; **GM-CSF**, granulocyte macrophage colony stimulating factor; **GRO**, growth regulated oncogene; **IBD**, inflammatory bowel disease; **IFN**, interferon; **Ig**, immunoglobulin; **IL**, interleukin; **MCP**, monocyte chemoattractant protein; **MIP**, macrophage inflammatory protein; **PBS**, phosphate buffer saline; **PCA**, principal component analysis; **TNF**, tumor necrosis factor.

INTRODUCTION

Beneficial effects of moderate consumption of wine have been evidenced mainly in relation to cardiovascular diseases[1-3]. The potential anti-inflammatory effects of red wine and its bioactive compounds, especially polyphenols, is the subject of continuous investigations. Wine polyphenols could contribute to the modulation of the inflammatory response as seen in some experiments with individual wine phenolic compounds, wine phenolic extracts, and red wine[4-6]. In addition, studies with polyphenols from other sources have proven their protective effects in inflammatory response[7] to such an extent that some authors have even proposed phenolic compounds as an alternative natural approach to prevent or treat chronic inflammatory diseases[8]. However, the anti-inflammatory effects of polyphenols are not fully understood, particularly at the intestinal level, and most of the molecular mechanisms related to their benefits remain largely unknown. So far, studies investigating the effects of wine-inherent polyphenols on intestinal inflammation have mainly been performed by using colonic cell models of inflammation[6, 9]. Therefore, *in vivo* studies are needed to further understand the mode of action of wine polyphenols in intestinal immune response.

Inflammation is a type of nonspecific immune response that defends the body against the constant threat of microorganisms and chemical substances from the surrounding environment. Specifically, chronic inflammation at gut level results from alterations of the gut homeostasis, in response to genetic and/or environmental triggers. All components of the gut, including the epithelial barrier, the mucosal immune system, and stromal/supportive cells, participate in this intestinal immune response. The exchange of regulatory signals, via the production of immune mediators (cytokines, growth factors, etc.) facilitates and amplifies cell interactions and/or activates inflammation[10]. These mediators, including immunoglobulins, cytokines, chemokines and growth factors, play an important role in the modulation of the intestinal immune system. In fact, chronic intestinal inflammation pathogenesis primarily implies

a dysfunction of the intestinal mucosa and an overproduction of pro-inflammatory mediators associated with an immune dysregulation[11].

Among immune compounds regulating mucosal inflammatory responses, cytokines have received special attention. They can be divided into two categories: those that up-regulate (pro-inflammatory) and those that down-regulate (anti-inflammatory) the inflammatory response. Inflammation is characterized by the interplay between pro- and anti-inflammatory cytokines[12]. Several authors have studied the role of various cytokines in the pathogenesis of inflammatory bowel diseases (IBDs), and the possibility that these mediators represent rational targets for therapeutic intervention. IBDs, including Crohn's disease and ulcerative colitis, are characterized by an abnormal response of the intestinal immune system against components of the normal enteric microbiota, leading to abdominal pain and chronic diarrhea[13]. Although the etiology and pathogenesis of IBDs are not yet clear, an imbalance of pro-inflammatory cytokines seems to play an important role in modulating inflammation[14,15]. The quantification of classical inflammation mediators (e.g. IL-1ß, IFN-γ, TNF-α, and IL-8) is considered a good approach to ascertain the status of the inflammation processes. Moreover, TNF-α released by monocytes/macrophages plays a key role in initiating the cascade of other pro-inflammatory cytokines, including IL-1ß, IL-6, and IFN-γ, which are important biomarkers in gastroenteric inflammation[13]. C-reactive protein[16] and fecal calprotectin[17,18] have been used widely as noninvasive markers of gut inflammation, including active IBD.

The aim of this study was to investigate the possible effects of moderate wine consumption on the gut immune status of healthy people through the study of a wide spectrum of fecal immune parameters, including several immunoglobulins, cytokines, chemokines and growth factors. Possible relationships between contents of these immune parameters and of phenolic metabolites present in feces as a consequence of wine consumption were explored.

MATERIALS AND METHODS

Red wines

Initially, different oenological parameters and practices, including grape variety and time of contact with seeds and skins were optimized at the winery, with the aim of obtaining a red wine with especially high polyphenol content (data not shown). Finally, a young red wine (Pinot Noir, vintage 2010) with a total phenolic content of 1758 mg of gallic acid equivalents/L was selected for the intervention study. Ethanol, pH, total acidity and volatile acidity were determined in the wine according to the International methods of the OIV (International Organization of Vine and Wine, 1990). The results were as follows: the wine pH was 3.52; alcohol degree: 13.8% v/v, total acidity: 6.45 g/L tartaric acid, and volatile acidity: 0.56 g/L acetic acid. Wine phenolic composition determined by UPLC-ESI- MS/MS is shown in Table 1.

Human fecal samples

Fecal samples collected in a controlled and randomized human intervention study carried out at the CIAL-Institute of Food Science Research (CSIC-UAM) according to a computer-generated random-number table[19] involving 42 "healthy" volunteers (34 case and 8 control subjects; 23 women and 19 men; age range 20–65 years), were selected for the present study. The participants were not suffering from any disease or intestinal disorder, and were not receiving antibiotics or any other medical treatment for at least 6 months before the start of the study or during the study (including the washout period). All the participants were fully informed about the study and gave written informed consent. The study was carried out according to the rules and approval of the Bioethics committee of Hospital "Ramón y Cajal" (Madrid, Spain).

The intervention study was divided into two consecutive periods: *a)* an initial washout period of 2 weeks (baseline) during which the volunteers did not consume any wine or any other alcoholic beverage and followed a low-polyphenols diet, and *b)* a period of 4 weeks during which the case volunteers consumed a daily intake of red wine (250 mL) of red wine over 28 days (intervention period), divided into two doses. During this latter period, participants also maintained the restrictions for any other alcoholic beverages and followed a low-polyphenols diet. The control group (n = 8) followed the same pattern as the case group (n = 34), with the exception that no wine was ingested during this 4-week period.

Each participant provided samples of feces at two points: *a)* after the washout period, and *b)* at the end of the study. Feces were immediately frozen and stored at -80 ºC awaiting analysis.

Preparation of fecal solutions for analysis of immune compounds

Fecal samples (~0.1 g) were suspended in 1 mL of PBS. After homogenization, the samples were centrifuged (14000 x g, 15 min, 4 ºC) and the supernatants (~200 μL) were collected.

Detection and quantification of immune compounds in the fecal samples

Fecal concentration of immunoglobulins (Ig) IgG1, IgG2, IgG3, IgG4, IgM and IgE, and cytokines, chemokines and growth factors, including interleukins (IL) 1_{β}, 2, 4, 5, 6, 7, 8, 10, 12(p70), 13, and 17, interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), growth-regulated oncogene-alpha (Gro-α), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1_{β} (MIP-1_{β}), granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF), were determined in fecal solutions by using the Bio-Plex Pro Human Isotyping assay and the Human Cytokine group I and II assay kits (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's protocol, in a Bioplex 200 system instrument (Bio-Rad Laboratories, Hercules, CA,

USA). Sample determinations were carried out in duplicate. Standard curves of each analyte were performed from triplicates of each assayed concentration on each plate, with the Bio-Plex Manager 6.0 software (Bio-Rad Laboratories, Hercules, CA, USA). The variation coefficient for the standard curves between plates was less than 10%.

The concentration of IgA in the fecal solution supernatants was determined using a specific enzyme-linked immunosorbent assay (ELISA) kit (Cusabio Biotech Co., LTD., Wuhan, China), according to the manufacturer's instructions.

Quantification of calprotectin

Fecal calprotectin values were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) Kit (Calpro, Lysaker, Norway). The standard curve of calprotectin was obtained from triplicates of each assayed concentration and fit to a 4-parameter curve model according to manufacturer's recommendations.

Analysis of phenolic metabolites

Phenolic metabolites were determined in the fecal samples as previously reported[19], although in this paper data were arranged in different manner according to the groups resulted from the analysis of immune compounds. Briefly, fecal samples were thawed at room temperature, homogenized and weighted (1.0 g) in 15 mL sterile conical tubes. 10 mL of sterile saline solution (NaCl 0.9%, Fresenius Kabi, Spain) spiked with the internal standard (4-hydroxybenzoic 2,3,5,6-d_4 acid) was added, vortered and centrifuged (10 min, 10000 rpm, 4 ºC) twice. The supernatant (fecal solution) was filtered (0.22 μm) and diluted with acetonitrile (1:4, v/v, acetonitrile/fecal solution). 2.0 μL of sample were injected onto the chromatographic system. Fecal samples were analyzed by an UPLC-ESI-MS/MS method validated

for the screening of more than 60 microbial derived phenolic metabolites including phenols, valerolactones and mandelic, benzoic, hippuric, phenylacetic, phenylpropionic, valeric and cinnamic acids. The chromatographic conditions and MS/MS parameters (cone voltage, collision energy and MRM transition) of the phenolic compounds targeted in our study were previously reported[20]. Fecal samples were analyzed in duplicate.

Statistical analysis

The statistical analysis was performed using STATISTICA program for Windows, version 7.1 (Statsoft Inc., 2005, www.statsoft.com). As the data were not normally distributed, medians and interquartile ranges (IQR) were calculated for all the groups. Principal components analysis (PCA) was used for exploration of the data and tentative classification of the case volunteers. For assessing significant differences between low- and high-cytokine-values subgroups before intervention, the nonparametric Mann-Whitney U test was performed. For assessing significant differences before and after the intervention period for both the intervention group – either as a whole group (n = 34) or divided into low- (n = 28) and high- (n = 6) cytokine-values subgroups – and the control group (n = 8), the nonparametric Wilcoxon test was applied. In both cases, a value of P = 0.05 was fixed for the level of significance of the tests. Finally, Pearson´s correlation coefficients among the different immune parameters and between immune parameters and the total content of phenolic metabolites were calculated.

RESULTS

Changes in the concentration of the immune parameters after wine consumption

Without doubt, selection of the red wine for the intervention study is a key step in order to achieve maximum effects of wine active compounds *in vivo*. The wine used in this study was especially rich in flavan-3-ols, a class of compounds that represented 60% of the phenolic compounds identified (Table 1). Total flavan-3-ol content (270 mg/L) in this wine was relatively higher in comparison to other red wines[21]. After the wine intervention study, fecal samples from all the subjects included in the intervention trial (n = 42) were available for the immune parameters study. A high inter-individual variability in the fecal concentration of the immune compounds analyzed in this study was observed either before or after the wine intervention (Table 2). Globally, no statistically significant differences in any fecal immune parameter were observed before or after wine consumption, except for IL-13 (P = 0.044), when the whole intervention group (n = 34) was considered (Table 2).

PCA was applied to all samples (before and after the intervention period), from both the intervention and control groups, in order to obtain a possible grouping of the volunteers according to their immune compound values. The first principal component (PC1) explained 60.7% of the total variance whereas the second principal component (PC2) only explained a 16.4% of the total variance (Figure 1). PC1 was highly correlated with the cytokine variables: G-CSF (-0.99326), IL-2 (-0.98931), IL-4 (-0.98626), TNF-α (-0.98531), IL-6 (-0.98453), IL-12(p70) (-0.98369), IFN-γ (-0.98039), GM-CSF (-0.97632), IL-17 (-0.96915), IL-10 (-0.96162), IL-8 (-0.94341), MCP-1 (-0.93860), IL-5 (-0.93454). Interestingly, the PCA representation allowed the detection of a volunteers' subgroup within the intervention group which was characterized by significantly high concentrations for most of the cytokines before starting wine consumption (Figure 1A; Table 3), a fact that was in agreement with their low PC1 values. As a consequence, these 6 volunteers exhibiting high fecal cytokine values before intervention were considered as a subset (high-cytokine-values subgroup) clearly differentiated from the rest (low-cytokine-values subgroup, n = 28) of the intervention group.

Then, the concentration of calprotectin in the samples of the 6 volunteers of the high-cytokine-values subgroup was determined in order to detect a potential intestinal inflammation. Results revealed that all the volunteers from the high-cytokine-values subgroup exhibited normal fecal calprotectin values (2.07–37.73 mg/kg feces) [22,23] and inflammatory diseases were discarded.

When comparing the values obtained at the beginning and at the end of the study, no significant differences in the concentrations of either immunoglobulins (data not shown) or cytokines, chemokines and growth factors (Table 3) were found for the low-cytokine-values subgroup (n = 28) or for the control group (n = 8). However, significant differences were found for 16 of the 24 analyzed immune compounds (IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12(p70), IL-13, IL-17, IFN-γ, Gro-α, MCP-1, MIP-1ß, TNF-α, G-CSF and GM-CSF) in the samples of the high-cytokine-values subgroup, after wine consumption (Figure 2; Table 3). Interestingly, among them, the medians of TNF-α, IL-6 and IFN-γ decreased 90-, 58- and 30-fold, respectively, after the intervention period (Table 3).

Relationships among immune compounds

Pearson's correlation coefficients for cytokine values were determined for the samples of the two subgroups in which the case population was classified. Positive and significant correlations ($P < 0.01$) among the different cytokines were found for both the high- and low-cytokine-values subgroups before intervention (data not shown). In the high-cytokine-values subgroup, the highest correlations were established among the pro-inflammatory cytokines IL-2, IL-6, and IL-8, and also among IL-2, IL-4 and C-GSF. Correlations among cytokines in the low-cytokine-values subgroup were notably lower (data not shown). In both subgroups the cytokines with the lowest correlations were IL-1ß and IL-7.

Relationships between immune compounds and fecal phenolic metabolites

A total of 35 phenolic metabolites including mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, valeric acids, valerolactones and cinnamic acids were found in the fecal samples used in this study[19]. To better summarize changes after wine consumption and investigate a possible relationship with immune compounds, the sum of the concentration of all metabolites (total phenolic metabolite content) was calculated considering the subgroups exhibiting high and low cytokine content before wine intervention. For both groups, the range of variation (min-max interval) for the total fecal phenolic slightly moved forward after wine intake: from 122.2-501.1 µg/g to 169.2-864.6 µg/g (before and after wine intake, respectively) for the high-cytokine-value subgroup (n = 6) and from 46.2-1307.9 µg/g to 111.8-1364.5 µg/g (before and after wine intake, respectively) for the low-cytokine-value subgroup (n = 27, after excluding one volunteer not considered in our previous study[19]). Pearson´s correlation coefficients between the content of immune parameters and of fecal phenolic were calculated separately for both the high- and low-cytokine-values subgroups, before- and after- wine consumption. For the high-cytokine-values subgroup, and for data before the wine intervention, negative and significant correlations ($P < 0.05$) were found between the total content of phenolic metabolites and the immune markers IFN-ϒ ($r = -0.892$), IL-8 ($r = -0.892$) and IL-6 ($r = -0.876$) (Figure 3). Interestingly, after wine intake, no significant correlations were found except for IL-8 ($r = 0.832$), but this fact was due to the high value obtained for the subject V25 (Figure 3A). For the low-cytokine-values subgroup, no significant correlation between any cytokines and total phenolic metabolites content was found neither before or after wine consumption (see Figure 3 for data corresponding to IFN-ϒ, IL-8 and IL-6).

DISCUSSION

During recent years, the potential immunomodulatory and anti-inflammatory effects of wine have been investigated. These studies carried out so far have been performed in serum as biological fluid. Chiva-Blanch *et al.*[4] studied the seric concentrations of some inflammatory-related molecules after administration of red wine, dealcoholized red wine or gin to high-cardiovascular-risk male volunteers, and found that IL-6 concentration was significantly lower after the intake of wine or dealcoholized wine in comparison to that of gin. This finding suggested that wine compounds other than ethanol would be responsible for the IL-6 decrease. Another human intervention study with 35 healthy women who consumed white or red wine (~20 g ethanol/day) during two periods of 4 weeks also reported decreases in serum inflammatory biomarkers, such as C-reactive protein, and in IL-6 and MCP-1 after wine consumption[24].

To our knowledge, this is the first paper to investigate the effects of wine *in vivo* on intestinal immunomodulation and inflammation in healthy volunteers. Our experimental design tried to assess changes in as many immune markers as possible, in order to have the most complete overview of the immune status. The results of the study showed that moderate wine consumption for 4 weeks resulted in a significant decrease in the concentration of several cytokines among the volunteers of the subgroup that exhibited high cytokine values before the intervention (Table 3, Figure 2). No significant differences in the cytokine values were observed among the rest of the intervention volunteers after wine intake (Table 3, Figure 2). None of the control group's volunteers (no wine intake) exhibited high cytokine values in their initial samples, which could be due to its low number (n = 8) in comparison to that of the intervention group (n = 34).

Previous studies have shown anti-inflammatory effects of wine extracts in colonic cell models[6,9,25]. Nunes *et al.*[9] studied the effect of a red wine extract on a cytokine-stimulated HT-29 cell model and demonstrated that the extract significantly reduced the cytokine-induced overproduction of IL-8 in a

dose-dependent manner. Angel-Morales *et al.*[26] used a red wine extract on an LPS-induced inflammation model of human colon CCD-18Co fibroblast cells and observed a decrease in the LPS-induced production of the pro-inflammatory cytokines IL-6 and TNF-α. Another study assessed the effects of polyphenols on intestinal inflammation by using Caco-2 Transwell model and results confirmed that polyphenols from different sources, including red wine polyphenols, moderately modulate intestinal inflammation[27]. As red wine polyphenols comprise a great diversity of compounds, including phenolic acids, flavan-3-ols and anthocyanins, studies with individual wine phenolic compounds have also been performed in order to specifically study the influence of the chemical structure on their anti-inflammatory properties. Bognar *et al.*[5] studied the anti-inflammatory effect of malvidin, a major red wine polyphenol, in an inflammation cell model, and concluded that this compound was responsible, at least partially, for the positive effects of moderate red wine consumption on inflammation-mediated chronic maladies such as obesity, diabetes, hypertension and cardiovascular disease. Other authors have studied the effect of polyphenols from different sources on experimentally induced gut inflammation *in vitro* and *in vivo.* Sergent *et al.*[11] tested different representative dietary phenolic compounds on an *in vitro* model of inflamed human intestinal epithelium and demonstrated that genistein and (-)-epigallocatechin-3-gallate provoked a decrease in IL-6 and IL-8 overproduction. Larrosa *et al.* and Sánchez-Fidalgo *et al.*[28,29] founded significant reductions in the expression of IL-1ß, IL-8, IFN-γ and TNF-α, or IL-1ß and TNF-α, respectively, when administering hydrocaffeic acid or resveratrol, respectively, to the DSS-treated rats used as a colitis model. Although most of these studies were conducted with the original phenolic forms present in wine or food, their derived metabolites may exhibit similar or enhanced anti-inflammatory properties[28]. In this context, a parallel study, focused on the phenolic metabolites present in the same set of fecal samples assayed in this paper, has revealed a great variability among subjects in the fecal content of phenolic metabolites (including benzoic,

mandelic, hippuric, phenylacetic, phenylpropionic, cinnamic and valeric acids, phenols, valerolactones, and others)[19]. Red wine phenolic content might be directly associated with the decrease observed in several cytokines after wine intake by the volunteers showing a high-concentration cytokine profile before the intervention, since it was found a significant increase of phenolic metabolites in the fecal profile after wine consumption. In this work, we demonstrated negative and significant correlations among the cytokines IFN-ϒ, IL-8 and IL-6 and the total content of phenolic metabolites for the high-cytokine-values subgroup before wine intake. This negative correlation between the content of certain cytokines and the content of phenolic metabolites at initial stages of gut inflammation (i.e., for the high-cytokine-values subgroup) suggested an anti-inflammatory action by phenolic metabolites at the epithelial level. In accordance with our findings, Chun et al.[30] found that intake of total flavonoid and also individual flavonol, anthocyanidin, and isoflavone intakes were inversely correlated with serum C-reactive protein in a large study (n = 8335). Also, in another study with apparently healthy subjects (n = 2115), Lanberg et al.[31] examined the association between intake of total and six flavonoid subclasses as well as flavonoid-rich food sources in relation to plasma biomarkers of inflammation (including IL-6, IL-18 and C-reactive protein among others), and found negative correlations between several flavonoid classes and the pro-inflammatory cytokine IL-18. Cytokines play a key role in regulating the intestinal immune response and inflammation which is characterized by the loss of the physiological equilibrium between pro-and anti-inflammatory cytokines[12]. In our study, there was a significant decrease in the concentration of most of the tested cytokines after wine intake by the volunteers' subgroup characterized by high cytokines' concentration in the initial sample. The decrease was especially noticeable in relation to those cytokines (TNF-α, IL-6 and IFN-γ) that promote initial inflammation. Elevated values of fecal IFN-γ and TNF-α have been observed in fecal samples from patients with bacterial diarrhea[32] while

increased concentrations of some pro-inflammatory cytokines, mainly TNF-α, IL-1ß and IL-6, have also been detected in feces of IBD patients[33].

In summary, this study with healthy hosts showed, for the first time, that moderate consumption of a phenolic-rich red wine modulates the intestinal inflammatory response *in vivo*. This effect that could be attributed, at least partially, to the phenolic metabolites derived from wine polyphenols, may be particularly beneficial for hosts with an initial (and still non-symptomatic) stage of gut inflammation.

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

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Table 1. Phenolic composition (mg/L) of the Pinot Noir wine used for the intervention study.

Benzoic acids	
Gallic acid	27.30±0.20
Protocatechuic acid	3.88±0.01
3-O-Methylgallic acid	1.06±0.06
4-Hydroxybenzoic acid	0.57±0.01
Vanillic acid	1.85±0.03
Syringic acid	2.30±0.13
Benzoic acid	1.14±0.06
Salicylic acid	0.22±0.00
Phenols	
Phloroglucinol	0.33±0.03
Tyrosol	31.40±1.40
Dihidroxiphenylpropan-2-ol	0.30±0.05
Cinnamic acids	
Caffeic acid	6.97±0.26
p-Coumaric acid	1.39±0.02
Ferulic acid	0.22±0.02
Coutaric acid	8.64±0.01
Caftaric acid	4.98±0.33
Stilbenes	
Resveratrol	7.12±0.29
Flavanols and others	
(+)-Catechin	51.60 ±1.70
(-)-Epicatechin	34.90 ±2.90
Procyanidin B1	79.10 ±0.90
Procyanidin B2	44.70±0.60
Procyanidin B3	16.00±1.00
Procyanidin B4	12.90±0.30
Procyanidin B5	2.67±0.01
Procyanidin B7	5.75±0.15
Procyanidin C1	14.00±0.40
Other trimers	7.96±1.05
Flavonols	
Quercetin	1.92±0.01
Myricetin	0.70±0.03
Anthocyanins	
Delphinidin-3-*O*-glucoside	2.58±0.11
Cyanidin-3-*O*-glucoside	0.76±0.04
Petunidin-3-*O*-glucoside	4.06±0.13
Peonidin-3-*O*-glucoside	18.90 ±2.00
Malvidin-3-*O*-glucoside	36.70 ±3.40

Table 2. Median (IQR) values of the immune parameters analyzed in the fecal samples provided by the 34 case volunteers before and after wine intake.

Case group (n=34)					
Cytokines (ng/g feces)			*Immunoglobulins*		
	before	*after*		*before*	*after*
IL-1$_\beta$	0.08 (0.02;0.19)	0.04 (0.01;0.12)	**Ig G1 (µg/g feces)**	1.09 (0.51;9.16)	2.55 (0.64;5.60)
IL-2	0.00 (0.00;1.87)	0.07 (0.00;0.57)	**Ig G2 (µg/g feces)**	91.71 (27.96;206.5)	111.09 (31.13;175.79)
IL-4	0.05 (0.03;0.31)	0.06 (0.03;0.22)	**Ig G3 (µg/g feces)**	0.98 (0.29;3.39)	1.65 (0.51;2.99)
IL-5	0.06 (0.01;1.50)	0.02 (0.01;0.71)	**Ig G4 (µg/g feces)**	0.10 (0.00;0.77)	0.18 (0.06;0.59)
IL-6	0.09 (0.07;2.01)	0.14 (0.05;1.01)	**Ig M (mg/g feces)**	0.05 (0.03;0.14)	0.06 (0.02;0.13)
IL-7	0.02 (0.01;0.05)	0.01 (0.01;0.02)	**Ig A (mg/g feces)**	19.33 (13.79;24.93)	21.13 (12.52;27.43)
IL-8	0.05 (0.00;0.12)	0.01 (0.00;0.10)			
IL-10	0.08 (0.04;0.81)	0.09 (0.04;0.69)			
IL-12(p70)	0.46 (0.11;25.61)	0.36 (0.16;10.06)			
IL-13	0.006 (0.006;0.019)	0.006* (0.006;0.012)			
IL-17	0.84 (0.38;14.11)	0.98 (0.43;4.85)			
IFN-γ	6.45 (2.67;214.83)	5.27 (0.18;179.46)			
GRO-α	1.21 (0.60;1.55)	0.99 (0.45;1.31)			
MCP-1	0.50 (0.10;4.38)	0.32 (0.11;2.07)			
MIP-1$_\beta$	0.15 (0.00;0.42)	0.12 (0.00;0.32)			
TNF-α	0.24 (0.10;9.46)	0.49 (0.13;4.07)			
G-CSF	1.51 (0.77;10.01)	1.16 (0.73;7.40)			
GM-CSF	8.57 (6.12;15.85)	9.70 (7.26;14.67)			

*Significant differences in the median values after intervention (nonparametric Wilcoxon test).

Table 3. Median (IQR) values (ng/g feces) of the cytokines analyzed in the fecal samples provided by the 34 case and 8 control volunteers before and after wine intake.

	Wine intake				Control group (n=8)	
	High-cytokine values subgroup(n=6)		Low-cytokine values subgroup (n=28)			
	before	*after*	*before*	*after*	*before*	*after*
IL-1$_\beta$	0.13a (0.12;0.22)	0.17 (0.09;0.36)	0.05a (0.01;0.17)	0.03 (0.01;0.10)	0.01b (0.01;0.04)	0.01 (0.00;0.01)
IL-2	60.95a (46.55;75.54)	1.38 (0.14;2.72)	0.00b (0.00;0.09)	0.05 (0.00;0.50)	0.04b (0.00;0.25)	0.00 (0.00;0.10)
IL-4	10.51a (8.10;10.95)	0.31* (0.07;0.54)	0.04b (0.03;0.07)	0.06 (0.03;0.14)	0.04b (0.03;0.08)	0.03 (0.03;0.06)
IL-5	9.80a (6.50;13.73)	0.43* (0.03;0.76)	0.01b (0.01;0.35)	0.02 (0.01;0.45)	0.02b (0.01; 0.43)	0.01 (0.01;0.05)
IL-6	115.07a (78.38;142.80)	1.97* (0.25;3.44)	0.08b (0.06;0.15)	0.10 (0.04;0.64)	0.06b (0.02;0.93)	0.04 (0.03;0.11)
IL-7	0.01a (0.01;0.07)	0.01 (0.01;0.01)	0.02a (0.01;0.04)	0.01 (0.01;0.02)	0.01a (0.01;0.03)	0.01 (0.01;0.01)
IL-8	1.06a (0.62;1.31)	0.05* (0.00;0.12)	0.01b (0.00;0.10)	0.01 (0.00;0.09)	0.02b (0.00;0.09)	0.00 (0.00;0.04)
IL-10	39.46a (27.63;62.59)	0.54* (0.17;1.08)	0.05b (0.03;0.13)	0.08 (0.04;0.43)	0.11b (0.06;0.25)	0.05 (0.01;0.11)
IL-12(p70)	556.47a (441.56;682.70)	9.86* (0.80;17.71)	0.27b (0.10;0.94)	0.29 (0.15;4.12)	0.09b (0.03;5.57)	0.13 (0.07;0.83)
IL-13	0.20a (0.16;0.34)	0.02* (0.01;0.03)	0.01b (0.01;0.01)	0.01 (0.01;0.01)	0.01b (0.01;0.01)	0.01 (0.01;0.01)
IL-17	319.25a (260.75;417.16)	11.97* (1.45;25.23)	0.71b (0.33;1.80)	0.71 (0.48;4.28)	0.96b (0.05;4.15)	0.66 (0.44;0.96)
IFN-γ	3624.31a (2302.64;3952.46)	120.90* (15.99;272.89)	4.68b (1.91;15.29)	4.68 (0.01;93.03)	0.01b (0.01;50.01)	0.01 (0.01;6.19)
GRO-α	3.00a (2.60;3.31)	1.21* (0.88;1.65)	1.13b (0.56;1.40)	0.95 (0.18;1.29)	0.81b (0.01;0.97)	0.74 (0.33;1.14)
MCP-1	27.23a (23.54;34.02)	1.29* (0.29;1.94)	0.26a (0.09;2.42)	0.27 (0.10;2.52)	0.26b (0.10;0.66)	0.10 (0.07;0.52)
MIP-1$_\beta$	1.19a (0.76;1.87)	0.47* (0.12;0.65)	0.09b (0.00;0.27)	0.11 (0.00;0.27)	0.00b (0.00;0.13)	0.00 (0.00;0.11)
TNF-α	732.48a (561.02;904.56)	8.16* (0.81;20.28)	0.20b (0.09;0.51)	0.31 (0.12;3.13)	0.06b (0.02;1.48)	0.14 (0.03;0.23)
G-CSF	188.33a (123.79;209.41)	11.87* (2.16;17.76)	0.96b (0.64;2.78)	1.05 (0.72;6.31)	0.73b (0.48;3.74)	0.63 (0.46;1.42)
GM-CSF	180.79a (148.67;259.47)	17.18* (11.57;21.68)	7.53b (5.86;10.34)	9.31 (7.14;11.23)	7.06b (5.44;7.55)	7.24 (6.04;7.72)

a-b. Median values, before intervention, with different letters are significantly different ($P<0.05$) between groups (Mann-Whitney U test).

*Significant differences in the median values after intervention (nonparametric Wilcoxon test).

FIGURE CAPTIONS

Figure 1. Representation of the volunteers (squares) and controls (rhombus), before (A) and after (B) intervention, in the plane defined by the two principal components.

Figure 2. Line plots of variables TNF-α (A), IL-6 (B) and IFN-γ (C) before (rhombus) and after (squares) wine intervention.

Figure 3. Correlation plots between contents of the immune compounds IFN-ϒ (A), IL-8 (B) and IL-6 (C) and of phenolic metabolites present in feces before and after wine intervention for both the high- and low- cytokine-values subgroups.

Figure 1

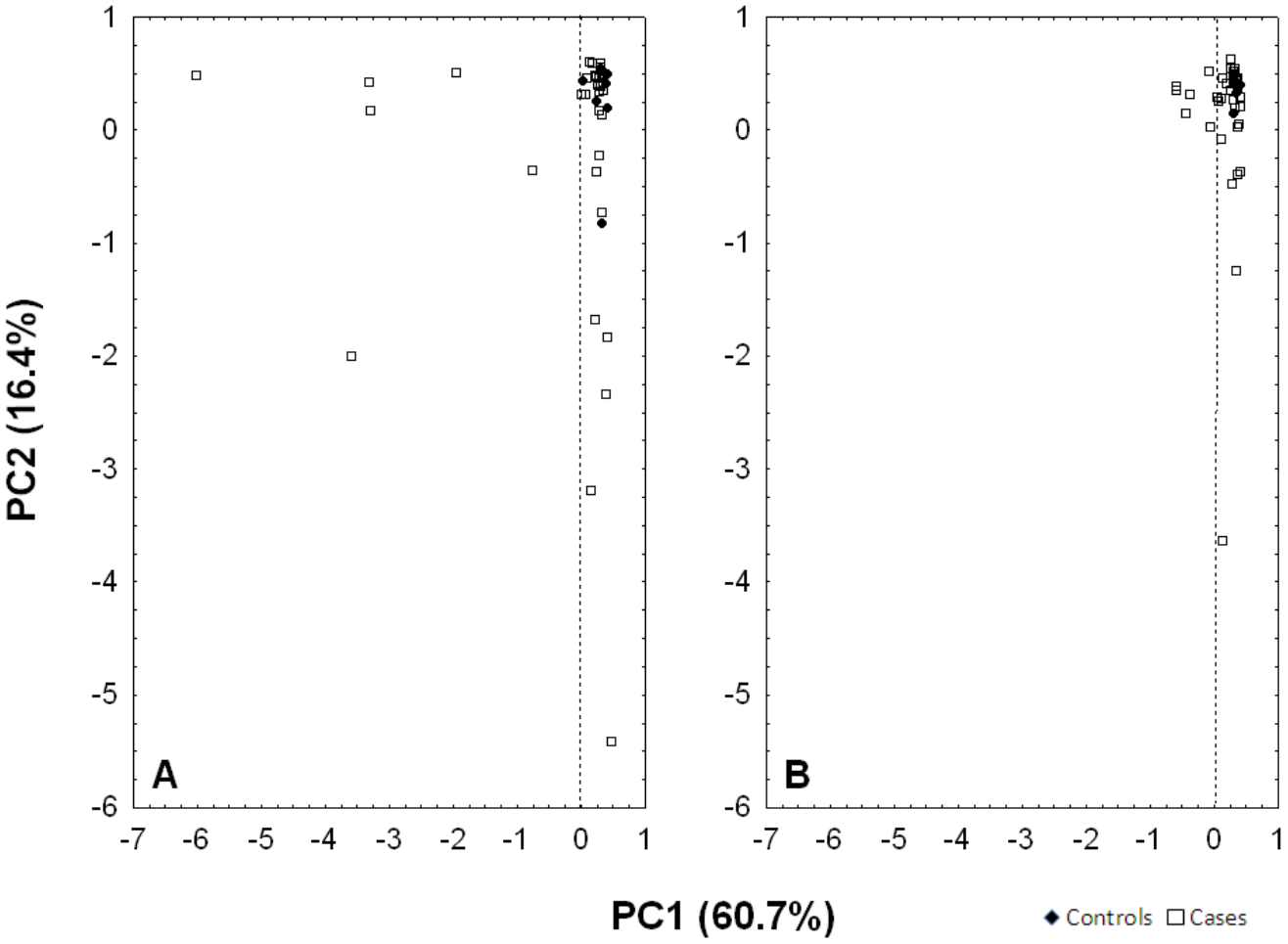


Figure 2

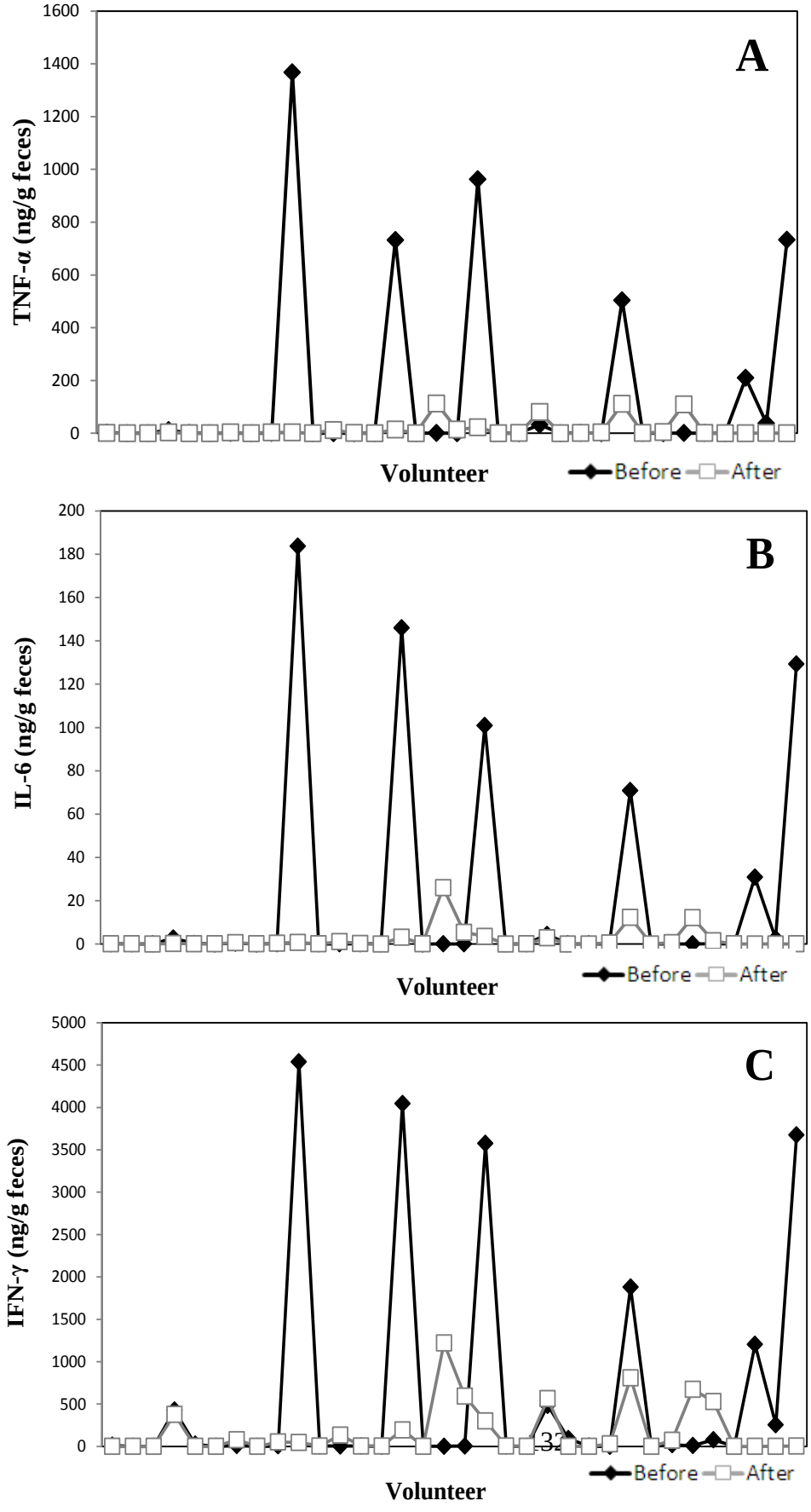
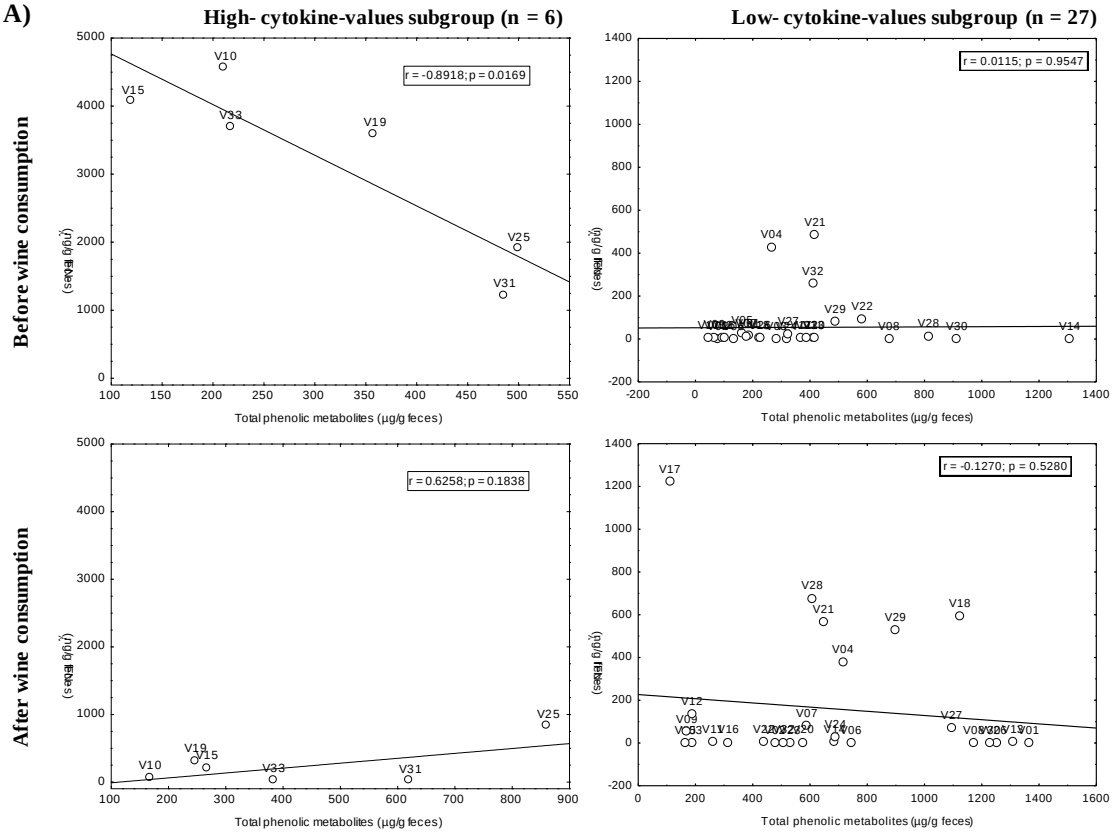
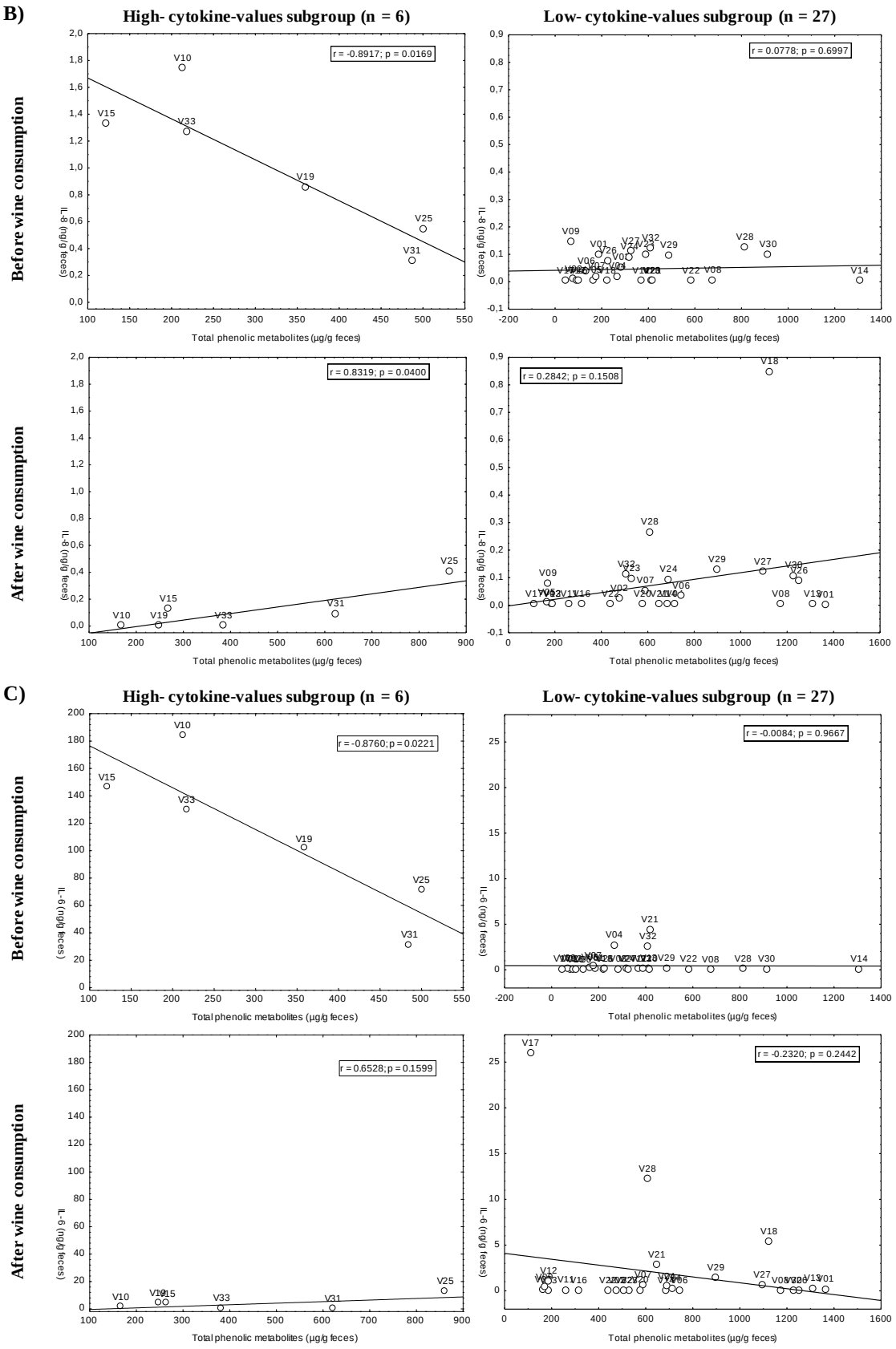

A
TNF-α (ng/g feces)
1600
1400
1200
1000
800
600
400
200
0
Volunteer
Before
After
B
IL-6 (ng/g feces)
200
180
160
140
120
100
80
60
40
20
0
Volunteer
Before
After
C
IFN-γ (ng/g feces)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Volunteer
Before
After

Figure 3


A)
High- cytokine-values subgroup (n = 6)
Low- cytokine-values subgroup (n = 27)
Before wine consumption
After wine consumption
r = -0.8918; p = 0.0169
r = 0.0115; p = 0.9547
r = 0.6258; p = 0.1838
r = -0.1270; p = 0.5280
Total phenolic metabolites (µg/g feces)
Total phenolic metabolites (µg/g feces)
Total phenolic metabolites (µg/g feces)
Total phenolic metabolites (µg/g feces)


B)
High- cytokine-values subgroup (n = 6)
Low- cytokine-values subgroup (n = 27)
Before wine consumption
After wine consumption
r = -0.8917; p = 0.0169
r = 0.0778; p = 0.6997
r = 0.8319; p = 0.0400
r = 0.2842; p = 0.1508
IL-6 (ng/g feces)
Total phenolic metabolites (µg/g feces)
C)
High- cytokine-values subgroup (n = 6)
Low- cytokine-values subgroup (n = 27)
Before wine consumption
After wine consumption
r = -0.8760; p = 0.0221
r = -0.0084; p = 0.9667
r = 0.6528; p = 0.1599
r = -0.2320; p = 0.2442
IL-6 (ng/g feces)
Total phenolic metabolites (µg/g feces)

Abstract

Polyphenols present in the wine may influence health modifying the metabolic activity and /or composition of gut microbiota. The aim of this study was to evaluate the impact of red wine in colonic metabolism using a novel *in vitro* Gastrointestinal Simulator (SIMGI), a dynamic model of human gut. To monitor the effects on microbial metabolism the ammonium and short chain fatty acids (SCFAs) concentrations were performed on samples from all colon compartments - ascending, transverse and descending. To verify the effects on microbial community composition, plate counts on specific growth media and a qPCR analysis were carried out. Significant differences were found for gallic acid, protocatechuic acid, 3-O-methylgallic acid, 4-hydroxybenzoic, 3,4-dihydroxyphenylpropionic acid, vanillic acid, syringic acid and salicylic acid during wine intake. Concurrently, a decrease in ammonium ion and an increase in butanoic acid production were observed. In turn, the microbiota metabolic activity was individual and compartment-dependent. At microbiological level, the main changes occurred in the ascending colon. In summary, the data highlight that red wine modulates the metabolic activity of colonic microbiota which could be physiologically relevant. In addition, the comparison of these results with a previous human intervention study has allowed validated the utility of SIMGI as gastrointestinal simulation model.

1. Introduction

The human large intestine is a dynamic microbial ecosystem inhabited by different bacterial species, which reaching their highest concentrations in the colon (up to 10^{12} cells per gram of feces) (Doré and Corthier 2010). The composition of the gut microbiota varies substantially among individuals. Consequently, each intestinal microbial community it is susceptible to change its microbial and metabolic profiles by dietary factors and diverse disease conditions (Salonen and De Vos 2014) . A well-balanced gut microbiota composition confers benefits to the host, whereas microbial imbalances are associated with metabolically mediated disorders (El Aidy, Van den Abbeele et al. 2013). The

consumption of some secondary metabolites, such as polyphenols, exerts significant effects on the intestinal environment, modulating the gut microbiota composition and probably their functional effects in mammalian tissues (Cardona, Andrés-Lacueva et al. 2013). These compounds are abundantly present in our diet in different food and beverages such as wine (Requena, Monagas et al. 2010).

To study the effect of dietary ingredients or food on gut microbiota, different approaches ranging from *in vitro* to *in vivo* studies can be used (Marzorati, Van Den Abbeele et al. 2011; Alminger, Aura et al. 2014). Due to their physiological relevance, investigations involve the use of animals or human trial provide the best models for studying the interactions of food components (e.g., polyphenols) with microbiota, however, they are so expensive, and in the case of human trial additional ethical constraints and limited opportunities to collect intestinal samples are found (Venema and van den Abbeele 2013). Therefore, even if they suffer from the absence of a physiological host environment, *in vitro* simulations, offer unique advantages. First, *in vitro* models allow focusing specifically on gut microbial activity and thus reduce some of the complexity caused by interactions with the host, for example, at the level of the gut epithelium. Second, microbial population and microbe-mediated metabolic effects can be dynamically monitored over time in different consecutive regions of human colon as in the novel Gastrointestinal Simulator model (SIMGI) (Barroso et al., submitted) that offer simulation of complex conditions present in the human gut.

Red wine, is one of the product diet with higher polyphenols content, mainly flavan-3-ols, as well as anthocyanins, catechins, and phenolic acids. The biological activity of wine polyphenols *in vivo* depends on their bioavailability that is, on whether they are absorbed and enters the blood circulation and tissues. Although polyphenol metabolism starts in the mouth and proceeds along the gastrointestinal tract, most of the dietary polyphenols reach the colon intact, where they are subjected

to the action of the gut microbiota, generating simpler phenolic derivatives which could present higher activity at a physiological level than the corresponding food precursors (Monagas, Urpi-Sarda et al. 2010). In this sense, several *in vitro* and *in vivo* studies have indicated that wine polyphenols selectively modulate the growth of some members of human microbiota (Tzounis, Vulevic et al. 2008; Viveros, Chamorro et al. 2011; Hidalgo, Oruna-Concha et al. 2012; Queipo-Ortuño, Boto-Ordóñez et al. 2012; Cueva, Sánchez-Patán et al. 2013; Kemperman, Gross et al. 2013). On the other hand, two recent human intervention studies have reported that red wine polyphenols also promotes changes in the phenolic metabolite profile of feces (Jiménez-Girón, Queipo-Ortuño et al. 2013; Muñoz-González, Jiménez-Girón et al. 2013).

Although some studies have investigated the effects of red wine polyphenols on the modulation of the human intestinal microbiota, to our knowledge current study is the first in simulate the red wine intake in an *in vitro* Gastrointestinal Simulator (SIMGI). This model comprised stomach, small intestine and vessels simulating ascending, transcending and descending parts of the human colon. Two independent SIMGI experiments were carried to evaluate red wine effects. The analysis of metabolic activity included the determination of microbial phenolic metabolites, SCFAs and ammonium. Changes in microbial populations were evaluated by microbial plate counting and qPCR. Furthermore, taking into consideration that the wine and selected volunteers were the same that in a previous human intervention study (Muñoz-González, Jiménez-Girón et al. 2013), the results obtained have enabled us, on the one hand, to make comparative study *in vivo* vs *in vitro*, and on the other hand, to validate the utility of SIMGI as gastrointestinal simulation model.

2. Materials and methods

2.1. Ingredients:

A young red wine (var. Pinot Noir, vintage 2010), kindly provided by Bodegas Miguel Torres S.A. (Catalonia, Spain) was used to carried gastrointestinal simulation in SIMGI. The wine phenolic composition is described in a recent paper from our investigation group (Muñoz-González, Jiménez-Girón et al. 2013). In addition, a synthetic wine composed of 4g/L tartaric acid and 12% ethanol, and subsequently adjusted to pH 3.6, was used to assess the effect of ethanol in metabolic activity of microbiota.

2.2. SIMGI model

2.2.1. SIMGI reactor

SIMGI is a computer controlled dynamic **SIM**ulator of the **G**astro**I**ntestinal tract consists of five successive reactors, simulating the stomach, small intestine, ascending (CA), transverse (CT), and descending colon (CD) regions. It maintains the temperature at 37ºC with a total retention tome of 76 h (Barroso et al., submitted). The colon vessels harbor a mixed microbial community and pH controllers (Unitronic Vision 120^{TM}) automatically maintain the pH in the range of 5.6 ± 0.2, 6.3± 0.2 and 6.8± 0.2 in the ascending, transverse and descending colons, respectively. In addition, all vessels were maintained under anaerobic conditions by continuously flushing of nitrogen.

2.2.2. Media used in SIMGI experiment

The nutritive medium contain arabinogalactan (1 g/L), pectin from apple (2 g/L), xylan (1 g/L), potato starch (3 g/L), glucose (0.4 g/L), yeast extract (3 g/L), peptone (1 g/L), mucin (4 g/L) and L-cysteine (0.5 g/L). All compounds were dissolved in 1 L of distilled water and sterilized at 121ºC for 15 min. For simulated the stomach medium, SIMGI medium was acidified with 37% HCl to reach

pH 2.0 for feeding the first vessel (small intestine). The pancreatic juice was prepared with 40 mL of a solution of 12 g/L $NaHCO_3$, 6 g/L oxgall dehydrate fresh bile and 0.9 g/L porcine pancreatine.

The fecal slurry (20%, w/v) to inoculated colon vessels was prepared from a fecal sample of healthy volunteer who had no received any antibiotic treatment in the previous 3 months of the experiment. To dilute the fecal sample, a sodium phosphate buffer (0.1 M, pH 7) containing 1g/L sodium thioglycolate as reducing agent was used.

2.2.3. SIMGI experimental set-up

In this study, a SIMGI set-up as described previously Barroso et al (submitted) was used by operated two independent experiments with fecal sample of two healthy volunteers that in a previous intervention study showed be high (volunteer 1) and moderate (volunteer 2) metabolizers of red wine polyphenols (Muñoz-González, Jiménez-Girón et al. 2013).

An initial stabilization period of two weeks after inoculation was applied to allow the intestinal bacteria to adapt to environmental conditions present in the colon vessels and to form a stable microbial community representative of the one present in the gastrointestinal tract (Barroso et al., submitted). After stabilization, to simulate intake of a glass of wine, 225 ml of young red wine (equivalent to a dose of ~ 405 mg of total polyphenols) was administered to the system in three times during one day (75 ml every 8 h). Then, six days of washout with nutritive medium was included in order to return to the initial conditions of stabilization (Van Dorsten, Peters et al. 2012). Moreover, in the case of one of the volunteer 2 further treatment with synthetic wine was carried out to assess the effect of ethanol in the composition and metabolic activity of the microbiota. Two intakes were carried out for each volunteer and/or treatment.

During the whole study, samples from the three colon vessels were collected at regular time points: stabilization period (SP), immediately prior to wine intake (-1), after 8 h of the beginning of wine intake (0), after completing wine intake (+1) and 24 h after the beginning of washout with nutrient

medium (+2). Prior to storage at -20ºC, samples were centrifuged at 10000 rpm, 10 min, 4ºC; pellets were used for DNA isolation and supernatants were filtered (0.22 μm) for further metabolic analysis (short chain fatty acid, ammonium, phenolic metabolites). Microbial plate count analysed were performed at the time of the sample.

2.3. Metabolic activity assessments

2.3.1. Short chain fatty acid (SCFA) Analysis

The SCFAs were determined by SPME-GCMS following the method developed by (Bianchi, Dall'Asta et al. 2011) with some modifications. Briefly, 290 µL of fermentation samples or calibration stock solutions were added with 10 µl of 10 gL-1 internal standard solution (2-methylvaleric acid) and with 30 µl of a 0.9M H_2SO_4 solution (pH=2). One hundred µL of the acidified sample were then transferred to a 20 ml hermetically closed vial. The extraction procedure was automatically performed by using a CombiPAL system (CTC Analytics AG, Zwingen, Switzerland) with a 50/30 µm DVB/CAR/PDMS SPME fibre of 2 cm length (Supelco, Bellefonte, PA). SPME conditions were as follows: extraction temperature 40 º C, extraction time 25 min and no salt addition. Temperature and time of desorption were 260 ºC for 2 min. Desorption was performed in the injector of the GC-MS system (Agilent 7890A, Agilent 5975C MS) in splitless mode for 2 min.

The chromatographic separation was performed in a DB-FFAP capillary column (30m x 0.25mm i.d. x 0.25 µm film thickness) (J&W, Agilent). Helium was used as carrier gas at a flow rate of 1mL/min. The oven temperature was initially held at 100ºC for 5 min, then increased at 5ºCmin-1 to 250 ºC and held for 12 minutes. The acquisitions were performed in scan (from 35 to 350 amu) and electronic impact mode (70eV). Other MS conditions were 270, 150 and 230 ºC for the transfer line, quadrupole and ion source respectively. Compound identification was carried out by comparison of retention times and mass spectra of the reference compounds with those reported in the mass

spectrum library NIST 2.0. Quantitative data were obtained by calculating the peak area of each compound in relation to that of the internal standard (2-methylvaleric acid). Calibration curves of each compound (acetic acid, propionic acid, 2-methylpropionic acid, butanoic acid, 2-methylbutanoic acid, pentanoic acid, hexanoic acid, octanoic acid and decanoic acid) were obtained diluting the original stock standard solution (5000 mgL^{-1} of all the SCFAs in the culture medium).

2.3.2. Ammonium determination

The ammonium concentration was determined using a selective pH/ion meter (7320 model WTW, Weilheim, Germany) coupled to an ammonium selective-ion electrode (WTW, Weilheim, Germany). Firstly, the equipment was calibrated using ammonium standard solution (10 g/L), at the concentrations of 0.1, 1, 10 and 100 mg $NH4^+$/L. Then, the test samples were prepared as follow: 0.5 ml of filtered SIMGI samples was diluted with 12 ml of deionized water. After adding 2% of sample conditioning solution (WTW, Weilheim, Germany) the sample was measurement immediately. All measurements were carried out al 25ºC. Ammonium ion concentration was expressed as mg NH_4^+/L.

2.3.3. Targeted Analysis of Phenolic Metabolites

The analysis of wine polyphenols and their metabolites were carried out by UPLC-ESI-MS/MS, as described previously (Muñoz-González, Jiménez-Girón et al. 2013). Briefly, SIMGI samples were defrosted, filtered through 0.22 μm, and spiked with the internal standard (IS) 4-hydroxybenzoic-*2,3,5,6-d*$_4$ acid (Sigma-Aldrich, St. Louis, MO). Stock solutions of phenolic standards (250 μg mL−1) were prepared by exact weighing of the analytes and dissolution with acetonitrile/water (1:4, v/v). Diluted solutions of 5 and 50 μg mL−1 were prepared to optimize the MS/MS parameters. According to their response, analytes were classified in five different groups (Sánchez-Patán, Monagas et al. 2011) (from highest to lowest response), and a stock standard pool solution was prepared by weighing individual compounds to achieve the following starting concentrations: 25, 50,

100, 200, and 500 μg mL^{-1}. Other solutions were prepared via serial dilutions and used in the generation of the calibration curves (11 different calibration levels from 10- to 10000-fold dilutions of the initial pool solution).

2.4. Microbial community analysis

2.4.1. Plate counting

Decimal dilutions in physiological solution of samples from the SIMGI were plated on four types of selective media as follow: Wilkins-Chalgren agar (BD) for total anaerobes, TSA (BD) for total aerobes, MRS (Pronadisa) for lactic bacteria and MacConkey agar (BD) for *Enterobacteriaceae*. Plates, with the exception of TSA, were incubated at 37 °C for 48 h in an anaerobic cabinet (BACTRON Anaerobic/Environmental Chamber, SHELLAB).

2.4.2. Bacterial DNA extraction:

Microbial DNA extraction of the samples taken from the AC, TC and DC compartments and from standard bacteria (*Escherichia coli* ATCC 25922 and *Bacteroides fragilis* DSM 2151) was performed using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following manufacturer´s recommended protocol. Concentration and quality of the purified DNA was measured in a NanodropTM spectrophotometer (Thermo Scientific, DE, USA). A260/280 and A260/230 values were greater than 1.8 and suitable for analysis. Before DNA extraction, a sample of each culture of the standard bacteria was serially diluted and cultured in specific media (TSA for *E.coli* and Modified Chopped Meat Medium Agar for *B. fragilis*), and colony forming units were counted.

2.4.3. Quantitative polymerase chain reaction (qPCR) analysis:

The amplification and detection of the bacterial DNA in the samples was performed on the Applied Biosystems® *ViiA™ 7* Real-Time PCR System using 384-well plates. Each amplification reaction was done in duplicate for each fermentation sample in a final volume of 10 µL that contained 5 µL of SYBER® Select Master Mix (Life Technologies, TX, USA), 200µL of each primer (10 µM), 600 µL of nuclease-free water purified for PCR (Sigma-Aldrich) and 4µL of DNA template. The amplification program consisted of one cycle at 50ºC for 2 min, one cycle at 95ºC for 10 min, 40 cycles at 95ºC for 30 s and 60 ºC 1 min, and finally one cycle of melting curve analysis for amplicon specificity at 95ºC for 15 s and 60 ºC for 1 min, increasing the ramp rate by 0.05 ºC/min until maintaining at 95ºC for 15 s. The 16S rRNA-targeting primers used in this study are listed in **Table 1.** Standard curves were determined with DNA isolated from selected bacterial strains: DNA from *Escherichia coli* ATCC 25922 and *Bacteroides fragilis* DSM 2151 were used for *E.coli* group and All bacteria and for Bacteroides group, respectively. In order to quantify bacterial groups, standard curves were generated from serial dilutions of a known concentration (100 ng/µL). Concentrations expressed in CFU/mL were also known by CFU counting in specific media. Standard curves were generated by plotting Threshold cycles (Ct) vs. bacterial quantity expressed as colony forming units (CFU)/mL.

2.5. Statistical analysis

The statistical methods used for the data analysis were as follow: *t* test for dependent samples to evaluate differences in means of CFU ml^{-1} in each compartment before and after intake; analysis of variance (ANOVA) was used to test the main effects of factors studied (time, compartment); and least significant difference (LSD) test was applied for means comparisons ($P < 0.05$) between different times. Principal component analysis (PCA) from matrix correlation was used to summarize

changes in all variables studied. All statistical analyses were carried out using the STATISTICA program for Windows, version 7.1 (StatSoft. Inc. 1984−2006, www.statsoft.com).

3. Results

3.1. Microbial Metabolism of Phenolic Compounds after red wine intake in SIMGI model

A qualitative comparison of phenolic metabolites profile of two volunteers during red wine intake in the SIMGI model was depicted in **Table 2**. A total of 28 microbial-derived phenolic metabolites, including mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenilpropionic acids, valeric acids, valerolactones and cinnamic acids, were detected. The presence in the samples of some of the main phenolic compounds in the red wine of study, such as flavan-3-ols and stilbenes it was also evaluated.

Regard to phenolic compounds, it was found that the levels of (+)-catechin and (-)-epicatechin increase rapidly in the CA and CT after the red wine administration, probably due to their high content in wine, whereas in the CD appears in a later stage. Following a similar pattern, resveratrol was detected in all vessels with the exception of CD from the volunteer 2, probably because it has been entirely metabolized by the microbiota of the transverse colon.

The initial microbial metabolism of wine flavan-3-ols, such as (+)-catechin and procyanidin, produced 1-(3',4'-Dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol (i.e. phenylpropanol), that subsequently is converted into 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (Monagas, Urpi-Sarda et al. 2010; Sánchez-Patán, Monagas et al. 2011; Sánchez-Patán, Cueva et al. 2012). These metabolites were mainly detected in CT and CD, reaching its highest level when the red wine intake was completed. The not detection of this compounds could be explained by their rapidly bioconversion toward others intermediate metabolites such as valeric acids, which follow a similar pattern formation, with the exception of 4- hydroxy-5-(3’-hydroxyphenyl)-valeric acid which only was detected in CD of volunteer 2.

Shortening of the side–chain length of valeric acids by subsequent β-oxidation reaction resulted in phenylpropionic, phenylacetic and benzoic acids. It is important to mention, that some of these compounds, such as 3-(4-hydroxyphenyl)-propionic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, phenylacetic acid and benzoic showed a high increase before intake of wine. Focusing on dihydroxylated forms of phenylacetic and phenylpropionic acids, point out that both reach the high increase at time point 0, +1 and +2 in CA, CT and CD respectively. The same trend with slight differences between volunteers was observed for benzoic acids. In particular, gallic acid levels in the CA increase rapidly 8 h after red wine administration, which is consistent with its presence in the wine. At the same time, a potential gallic acid metabolites, 3-*O*-methylgallic, pyrogallol and catechol/pyrocatechol were observed. It is important to notice that the two latter compounds could not be detected in CA and CD (only for pyrogallol) vessels of volunteer 2, suggesting fast conversion. In contrast, the other phenol compound, 4-methylcatechol, only was found in the CD of volunteer 2.

Regard to cinnamic acids, were only detected in the ascending colon, with the exception of *p*-coumaric acid which was also found in the transverse and descending colon.

Others microbe-derived metabolites, such 4-hydroxy-3-methoxymandelic and hippuric acids, mostly appeared after completing the red wine intake. As an exception hippuric acid was not detected in the CD vessel of any volunteers.

In summary, we can say that the phenolic metabolite formation during red wine intake in SIMGI model occurs sequentially in advancing the process of digestion, that is, ascending colon and subsequently in the transverse and descending colon.

In addition to polyphenols, red wine contains ethanol. In order to determine its effect on the metabolic activity of colonic microbiota, at the end of the red wine intake from one of the volunteers, and after a washout period of 6 days with nutrient medium, the experiment was repeated with

synthetic wine. The results relative to analysis of phenolic compounds showed that, the synthetic wine does not generate microbial phenolic metabolites (result not showed).

In order to quantify the ability of the microbiota of the two volunteers to produce phenolic metabolites during the different stages of wine intake, a sum of metabolites was carried out. As shown **Figure 1**, the intake of wine in volunteer 1 resulted in a significant increase of metabolites production in CA and CT. In contrast, a significant decrease was observed in CA for volunteer 2. Nevertheless, for both volunteers, the CT turned out to be the higher producer of metabolites. Based on these results we can say that volunteer 1 was clearly more metabolizer than volunteer 2.

On the other hand, it was attempted to elucidate whether there was a parallelism between red wine intake in a simulation model (SIMGI) and in a human intervention study, taking into account that wine and selected volunteers (n = 2), were the same (Muñoz-González, Jiménez-Girón et al. 2013). For this purpose, using data from the metabolites produced in the descending colon, the ratio Σ metabolites vol 1 / Σ metabolites vol 2 was calculated. The results before and after wine intake were 3.28 and 2.92 in the intervention study and 1.69 and 2.13 in the SIMGI, confirming that the volunteer 1 was more metabolizer than volunteer 2.

3.2. Ammonium formation

Ammonium is a by-product of normal protein metabolism produced by intestinal bacteria. **Figure 2** displayed the trends of ammonium formation in colon vessels of SIMGI during red or synthetic wine intake. For both volunteers tested, administration of red wine led to a gradual decrease in the ammonium concentration in all vessels, being more pronounced in the ascending colon, and becoming statistically significant in the case of volunteer 1. On the other hand, the synthetic wine resulted in a slight decrease in ammonium concentration in the ascending colon and an increase in transverse and descending colon, being more remarkable in the latter. In addition, for the two volunteers, it was also observed that, at the end of washout period, the ammonium concentration tend

to return to initial values. This fact becomes evident in the volunteer 2 for which the initial ammonium values were very similar for both treatments.

3.3. Short-chain fatty acid (SCFA)

An important metabolic activity of the colonic microbiota is the formation of short-chain fatty acids (SCFAs). In general, the stabilization period was characterized by a decrease in the total concentration of fatty acids in all compartments, whereas during the intake of red and synthetic wine concentrations remained fairly stable. Moreover, the total concentration of fatty acids was dependent on the compartment, being higher in the transverse and descending colon (results not shown). Regarding SCFAs profile, a different patter was obtained in each stage. As an example, **Figure 3** shows the results for one of the volunteers, finding that there were differences in the proportion of fatty acids between the stabilization period and different treatments. In general, the stabilization stage was characterized by a fairly uniform profile of fatty acids within each compartment. In contrast, red wine intake led to an increase in butanoic acid at the expense of a decrease in acetic and/or propionic acid. It is worth mentioning that the increase in the proportion of butanoic acid in the ascending colon occurs earlier than in the transverse and descending colon, which probably is related to the pattern of fluid transfer between compartments according SIMGI set up. On the other hand, in the case of treatment with synthetic wine the most remarkable effect was an increase in the proportion of propionic acid.

3.4. Effect of treatment in the colon microbial community of SIMGI

In order to compare the influence of red wine versus synthetic wine in some of aerobia and anaerobia groups of intestinal microbiota, counts before and after intake were carried out for all vessels (**Table 3**). In the case of red wine, a significant decrease after intake was observed for total aerobes and lactobacilli in ascending colon, whereas the latter group increased in transverse colon. Not

significant differences were found between vessels for any of the group tested. In contrast, synthetic wine produced significant decrease in counts of total anaerobe and lactobacilli in the ascending colon respect transverse and descending colon. Additionally, significant differences were observed in the initial counts of total aerobic and *Enterobacteriaceae* between vessels.

3.5. Microbial changes observed by qPCR:

As an example, **Figure 4** shows, for volunteers 2, the effects of red wine administration in the SIMGI for some representative groups of intestinal microbiota, including total bacteria, Bacteroides and *E. coli*. The results showed that only a significant decrease ($p < 0.05$) in all bacteria group occurred in ascending colon after red wine administration. In the case of Bacteroides group, although the same tendency was observed, it was not significant. Therefore, it can be said that, in general, the microbial groups tested remains fairly stable through the red wine administration.

3.6. Most noticeable/remarkable metabolic and microbial changes during red wine intake

Finally, in order to summarize the changes in all variables studied (microbial groups, phenolic metabolites, SCFAs and ammonium) as a consequence of red wine intake, a PCA was applied. **Figure 5** represents the planed defined by the first two principal components (PC1 and PC2) that resulted for the data from samples in at -1, 0, +1 and +2 time points in ascendens, transversum and descenden colon of the two volunteer studied. The first principal component (PC1), explaining 33.9% of the total variance, reflected overall changes occurring during time-course of intake of wine. This component was negatively correlated (loadings ≤ -0.7) with gallic acid, protocatechuic acid, 3-O-methylgallic acid, 4-hydroxibenzoic acid, 3,(3`,4`-dihydroxiphenyl)-propionic acid, vanillic acid, syringic acid, and salicylic acid. As can be seen in **Figure 5**, lower values of PC1, corresponding to higher concentrations of the compounds mentioned, were found after completed red wine intake (+1) in ascending colon, and subsequently, (+2) in transverse and descending colon. Surprisingly, a

return to positive values in this component was observed in ascending colon at time +2. The second principal component, which explained 20.0% of the total variance, showed differences within the same compartment, and was negatively correlated with 3,4-dihydroxiphenylacetic acid, catechol/pyrocatechol and butanoic acid. During red wine intake, important differences were observed between compartments for both volunteers, especially in ascending colon.

4. Discussion

In recent years, a growing number of studies have showed that potential health effects of dietary polyphenols are due, in a large extent, to phenolic metabolites derived from colonic metabolism of polyphenols (Requena, Monagas et al. 2010). This, coupled with the metabolic activity of the colonic microbiota is region-dependent (Barroso, Sánchez-Patán et al. 2013), implies that, from an *in vitro* level, the approach to study polyphenol-microbiota interaction should be done using simulation models that dynamically monitoring microbiological and metabolic changes in different parts of the colon. Bearing this in consideration, this study addresses, for the first time, the use of a novel gastrointestinal simulation model, the SIMGI, to evaluate wine intake effect on colonic microbiota metabolism. Recently it has been shown that after a stabilization period of two weeks with carbohydrate-based medium, this model allows obtain a stable and differentiated microbiota in each colon compartment, and therefore can be used as tool for studying the effects of diet or food components, in our case wine, on modulating the gut microbiota and its metabolic activity (Barroso et al., submitted).

In order to assess the metabolic activity of microbiota during wine intake, several parameters, including phenolic metabolites, SCFAs and ammonium formation were measured. In terms of phenolic profile, wine intake caused an increase the formation of some wine polyphenols, such as (+)-catechin, (-)-epicatechin, and resveratrol in the ascending and transverse colon, and subsequently in descending colon, in which case, an origin from procianidin degradation cannot be rule out (Monagas, Urpi-Sarda et al. 2010). In accordance with our results, (Van Dorsten, Peters et al. 2012)

found that administration of a red wine extract in a SHIME model, produced an initial increase of the polyphenols presents initially in the extract.

The initial microbial catabolism of wine polyphenols gave rise, mainly in transverse and descending colon, to several intermediate metabolites such as diphenylpropan-2-ol and 5-(3`,4`-dihydroxyphenyl)-γ-valerolactone, which has been reported as biomarker of flavan-3-ols metabolism by gut microbiota (Sánchez-Patán, Chioua et al. 2011). The not-detection of these metabolites in some compartments at any time point could be due to its rapid bioconversion to valeric acids. Subsequent biotransformations of these acids by oxidation reactions give rise to hydroxyphenylpropionic, hydroxyphenylacetic and hydroxybenzoic acids by successive loss of carbon atoms from the side chain. In general, the higher level of these acids was reached sequentially according to digestion process, that is, in an early stage (0) in ascending colon and subsequently in transverse (+1) and descending (+2) colon. In particular, significant differences were found for the content of gallic acid, protocatechuic acid, 3-O-methylgallic acid, 4-hydroxybenzoic, 3,4-dihydroxyphenylpropionic acid, vanillic acid, syringic acid and salicylic acid during wine intake. Similarly, in a recent human intervention study with the same wine (Muñoz-González, Jiménez-Girón et al. 2013) found that 3,5-dihydroxybenzoic acid, 3-O-methylgallic acid, vanillic acid, protocatechuic acid syringic acid significantly increase after red wine consumption. In the same way, a significant increase in three mentioned latter acids was also found by (Jiménez-Girón, Queipo-Ortuño et al. 2013) after red wine consumption. An origin from the microbial catabolism of anthocyanins, one the main polyphenols in wine, it has been reported for syringic and vanillic acids (Aura, Martin-Lopez et al. 2005; Keppler and Humpf 2005). On the other hand, a significant differences between compartments were observed for 3,4 dihydroxyphenylacetic acid and catechol/pyrocatechol, which were mainly produced in transverse and descending colon likely due to oxidase, esterase and decarboxylase enzyme activities of gut microbiota (Meselhy, Nakamura et al. 1997; Roowi, Stalmach et al. 2010). In turn, it is important to point out that these phenolic acids

could have important health benefits, since it has been reported that have, among others, antioxidant, anti-inflammatory, anti-proliferative, anti-cytotoxic and antimicrobial activities (Monagas, Urpi-Sarda et al. 2010)

An exception in phenolic metabolite profile during wine intake was found for the acids 3-(4-hydroxyphenyl)-propionic acid, 4-hydroxyphenylacetic, 3-hydroxyphenylacetic, phenylacetic and benzoic, which appear at high level before wine intake. An origin from fecal water of volunteer was discarded because the stabilization period is enough to wash out the initial phenolic content of the donor. A possible hypothesis it would be that these acids coming from colonic microbial fermentation of carbohydrates and proteins present in the nutrient medium (Russell, Hoyles et al. 2013).

In parallel with the above, red wine administration to the SIMGI led to a gradual decrease in ammonium production in all compartments, which might be due to both the situation of exhausting nitrogen sources as a consequence of superseded SIMGI medium by red wine (Chaikham, Apichartsrangkoon et al. 2012), and the ability of wine polyphenols to reduce colonic protein fermentation (Jacobs, Fuhrmann et al. 2012). It is noteworthy that lower proteolytic activities are usually associated with health-promoting effects (De Wiele, Boon et al. 2004). According with our results, several studies have reported that the red wine extract administration to the SHIME model led to a decrease in the ammonium concentration (Kemperman, Gross et al. 2013; Barroso, Van De Wiele et al. 2014). On the other hand, the red wine intake changed SCFA production by increasing the level of butanoic acid, most likely be interpreted to be favorable since this acid is associated with a variety of beneficial health effects such as inhibition of inflammation, decreasing oxidative stress and anticarcinogenic agent (Scharlau, Borowicki et al. 2009; Van Immerseel, Ducatelle et al. 2010).

On the microbiological level, the red wine intake led to a decrease in the counting of total aerobes and lactobacilli in ascending colon, as well as an increase of the latter in transverse colon which could be physiological relevant, taking into consideration that some lactobacilli, such as

Lactobacillus acidophilus and *Lactobacillus plantarum* IFPL935 are able to improve the butanoic acid production (Chaikham, Apichartsrangkoon et al. 2012; Barroso, Van De Wiele et al. 2014). On the other hand, qPCR results showed that addition of red wine exerted antimicrobial effects on the gut microbiota in the ascending colon, particularly for *Bacteroides* and total bacteria groups. The same tendency was observed by (Kemperman, Gross et al. 2013) and (Barroso, Van De Wiele et al. 2014) after feeding the SHIME with red wine extracts.

The wine, in addition to polyphenols, contains among its major components ethanol (Pozo-Bayón, Monagas et al. 2012). In order to assess the effect of this component on the metabolic activity of the colonic microbiota, a simulated intake of synthetic wine was performed. The results showed that the microbiota was not able to generate phenolic metabolites, except those mentioned above as derived from degradation of the components of the culture medium. Regarding other metabolic parameters tested, it was found that synthetic wine tends to increase the ammonium and propionic acid concentrations, probably due to the adaptation of the microbiota to a limitation in the carbohydrate source (Marzorati, Verhelst et al. 2010). In addition, a noticeable antimicrobial effect of synthetic wine against lactobacilli and total anaerobes was observed in ascending colon, likely to the higher rate of ethanol in this compartment according to SIMGI set-up. Overall these results show that the metabolic activity of the colonic microbiota is modified depending on the food source.

The combined learnings of *in vitro* gut models and human intervention studies are important to understand local and systemic bioactive properties of phenolic. Taking into in consideration, a comparison between results from this study and a previous human intervention study (Muñoz-González, Jiménez-Girón et al. 2013) with the same wine and volunteers was used to validate the utility of SIMGI as gastrointestinal simulation model. For this purpose, the metabolic activity of microbiota from each compartment during wine intake was assessed, finding that the final compartments, that is, transverse and descending colon, were the major metabolites producers for both volunteers. In addition, results evidenced a higher metabolic activity of the volunteer 1 than

volunteer 2, which is in accordance with the previous classification as high and moderate metabolizer, respectively, made by (Muñoz-González, Jiménez-Girón et al. 2013). Bearing this in mind, and using only the results of the descending colon for physiological similarity to the intervention study, the ratio Σ metabolites vol 1 / Σ metabolites vol 2 before and after wine intake was calculated, again confirming the metabolic superiority of volunteer 1. In particular, it was found, on the one hand that the total metabolites concentration in human intervention study was higher and, on the other hand, that more metabolites were generated in the SIMGI after red wine intake. These differences could respond, at least in part, to the intrinsic limitations of each type of study, so that, in the former case, the intake of wine coexists with other dietary polyphenols foods, while in the second case, the absence of the absorption process causes accumulative effect of phenolic metabolites after wine intake (Van Dorsten, Peters et al. 2012). Nevertheless despite these considerations, the results highlight the usefulness of SIMGI as gastrointestinal simulation model.

In conclusion the administration of wine in SIMGI generates changes in the metabolic activity of the colonic microbiota, which is determined by the production of various phenolic metabolites, as well as a decrease in ammonium ion and an increase in butanoic acid production. In turn, the microbiota metabolic activity was individual and compartment-dependent. Concurrently, intake of wine produced slight changes at microbiological level, mainly in the ascending colon. Furthermore, the results of this study have demonstrated the utility of SIMGI as simulation model to evaluate microbial metabolic changes taking place in different colon regions.

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FIGURE CAPTIONS

Figure 1. Sum of phenolic metabolites in ascending colon (CA), transverse colon (CT), and descending (CD) during red wine intake in SIMGI model. Blue bars refer to time immediately prior to wine intake (-1); red bars to 8 h after beginning of wine intake (0); green bars refers to 24 h after completing wine intake (+1); and purple bars refers to 24 h after beginning of washout with nutrient medium (+2). Means in all colons followed with different code letters are significantly different ($p<0.05$) according to LSD test. The letter "a" was assigned to the lowest value. n.s: not significant differences.

Figure 2. Concentration of ammonium ion in ascending colon (CA), transverse colon (CT), and descending (CD) during the SIMGI run with red and synthetic wine administration. Blue bars refer to time immediately prior to wine intake (-1); red bars to 8 h after beginning of wine intake (0); green bars refers to 24 h after completing wine intake (+1); and purple bars refers to 24 h after beginning of washout with nutrient medium (+2). Means in all colons followed with different code letters are significantly different ($p<0.05$) according to LSD test. The letter "a" was assigned to the lowest value. n.s: not significant differences.

Figure 3. Evolution in the proportion of fatty acids in the SIMGI during the stabilization period (SP) and intake of red and synthetic wine. (-1) refers to time immediately prior to red/synthetic wine intake; (0) refers to 8 h after beginning of red/synthetic intake; (+1) refers to 24 h after completing red/synthetic intake; and (+2) refers to 24 h after beginning of washout with nutrient medium. The data plotted for the red/synthetic wine intake is the outcome of the average of 2 replicates carried out for each treatment. Others acids include 2-methylpropanoic acid, 3-methyl butanoic acid, pentanoic acid, hexanoic acid, octanoic acid and decanoic acid.

Figure 4. Quantitative PCR results on total bacteria, Bacteroides and *E. coli* in ascending colon (CA), transverse colon (CT), and descending (CD) during the SIMGI run with red wine

administration. Blue bars refer to time immediately prior to wine intake (-1); red bars to 8 h after beginning of wine intake (0); green bars refers to 24 h after completing wine intake (+1); and purple bars refers to 24 h after beginning of washout with nutrient medium (+2). Means in all colons followed with different code letters are significantly different ($p<0.05$) according to LSD test. The letter "a" was assigned to the lowest value. n.s: not significant differences.

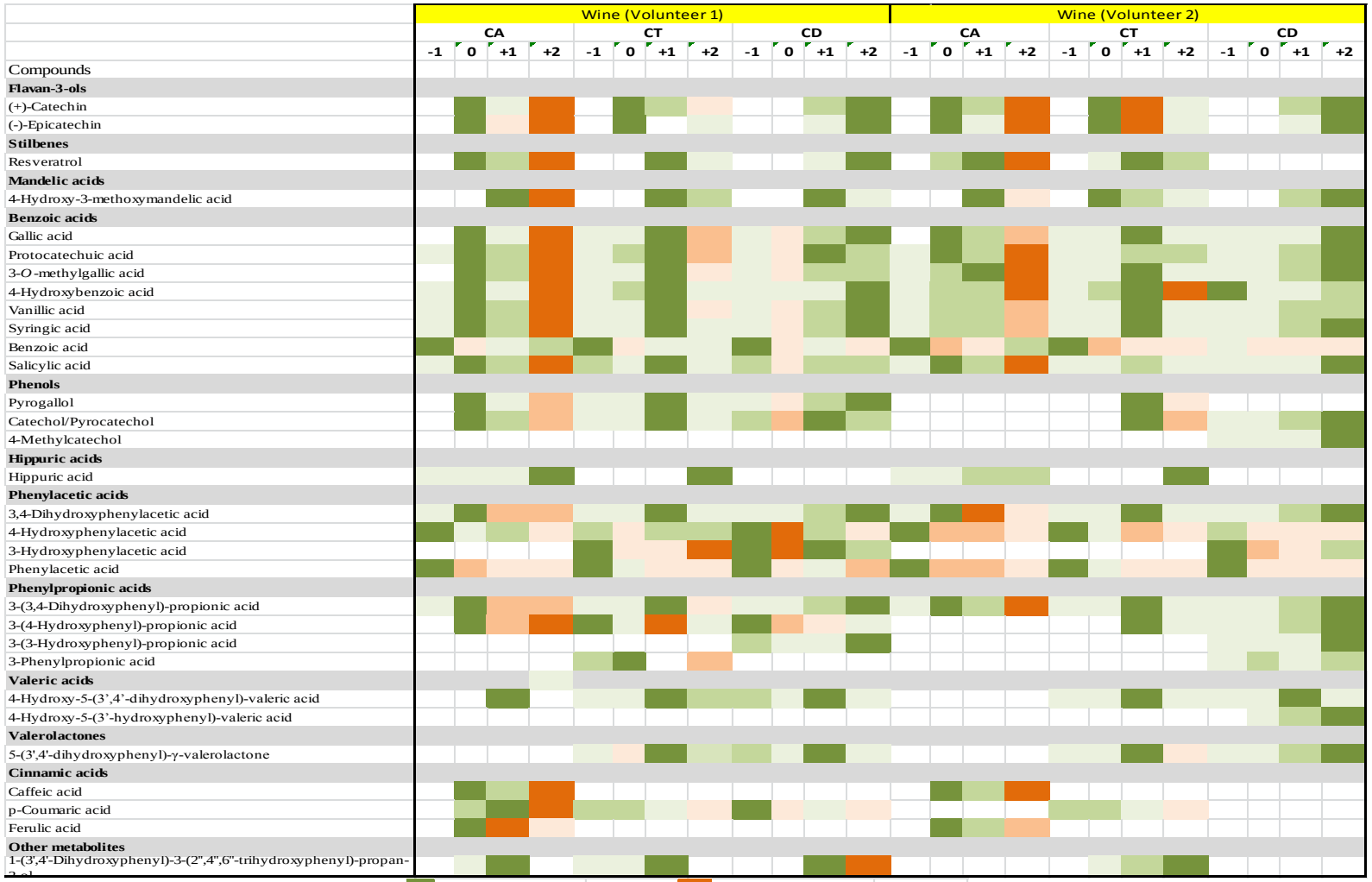
Figure 5. Representation of the samples in the plane defined by the first two principal components (PC1 and PC2) resulting from intake of red wine in ascending (A), transverse (B) and descending colon (C). Numbers represent volunteer 1 and 2.

TABLES

Table 1. Primers and references used for qPCR analysis

Target	Primer sequence (5'-3')	Ref.
All bacteria	CGG TGA ATA CGT TCC CGG TAC GGC TAC CTT GTT ACG ACT T	Furet et al. (2009)
Bacteroides	GAG AGG AAG GTC CCC CAC CGC TAC TTG GCT GGT TCA G	Guo et al. (2008)
E. coli	CAT GCC GCG TGT ATG AAG AA CGG GTA ACG TCA ATG AGC AAA	Huijsdens et al. (2002)

Table 2. Overview of observed changes in microbial phenolic metabolites production of two volunteers after red wine intake in the SIMGI model.



Colors in the table are obtained from the average of the values of concentration of compound/phenolic metabolites of the two replica of red wine intake performed for each volunteer. Colors in each cell denote qualitative increase (green) or decrease (orange) in phenolic production in the corresponding compartment (CA, CT, or CD) from the previous day. The presence of phenolic metabolites the day immediately prior to wine intake (-1) suggest suggests their origin from microbial degradation of the components of the nutrient medium

Table 3. Average plate count measurements (±SD), expressed in log CFU ml^{-1}, for the different microbial groups, treatment (red wine/synthetic wine), SIMGI compartments and periods.

Bacteria group	Time	Red wine			Synthetic wine		
		CA	CT	CD	CA	CT	CD
Total aerobes	-1	7,55±1,06	7,04±0,20	6,56±0,70	6,46±0,15 a	7,31±0,30 b	6,18±0,03 a
	+1	6,83±0,74*	6,76±0,70	6,41±0,73	6,28±0,47	6,72±0,73	6,53±0,85
Total anaerobes	-1	8,32±0,17	7,85±0,92	7,89±0,89	8,34±0,19	8,23±0,14	8,76±0,36
	+1	7,18±0,50	7,98±0,59	7,72±0,81	6,56±0,17* a	8,39±0,20 b	8,39±0,05 b
Enterobacteriaceae	-1	7,02±1,57	6,78±0,32	6,21±0,42	5,93±0,21 a	7,27±0,10 b	6,10±0,04 b
	+1	6,93±0,73	6,80±0,64	6,21±0,29	6,35±0,44	6,94±0,51	6,64±0,64
Lactobacilli	-1	6,90±1,64	6,29±0,91	5,61±1,00	6,18±0,04	6,27±0,02	6,21±0,05
	+1	6,11±1,65*	7,03±0,53*	6,22±0,47	4,98±0,03* a	6,17±0,07 b	6,09±0,07 b

*Mean values significantly different in CFU ml^{-1} before (-1) and after intake (+1). For a given microbial group analyzed, different lowercase letters denote significant differences ($P < 0.05$, from LSD test) between compartments.

FIGURE GRAPHICS

Figure 1.

Figure 2.

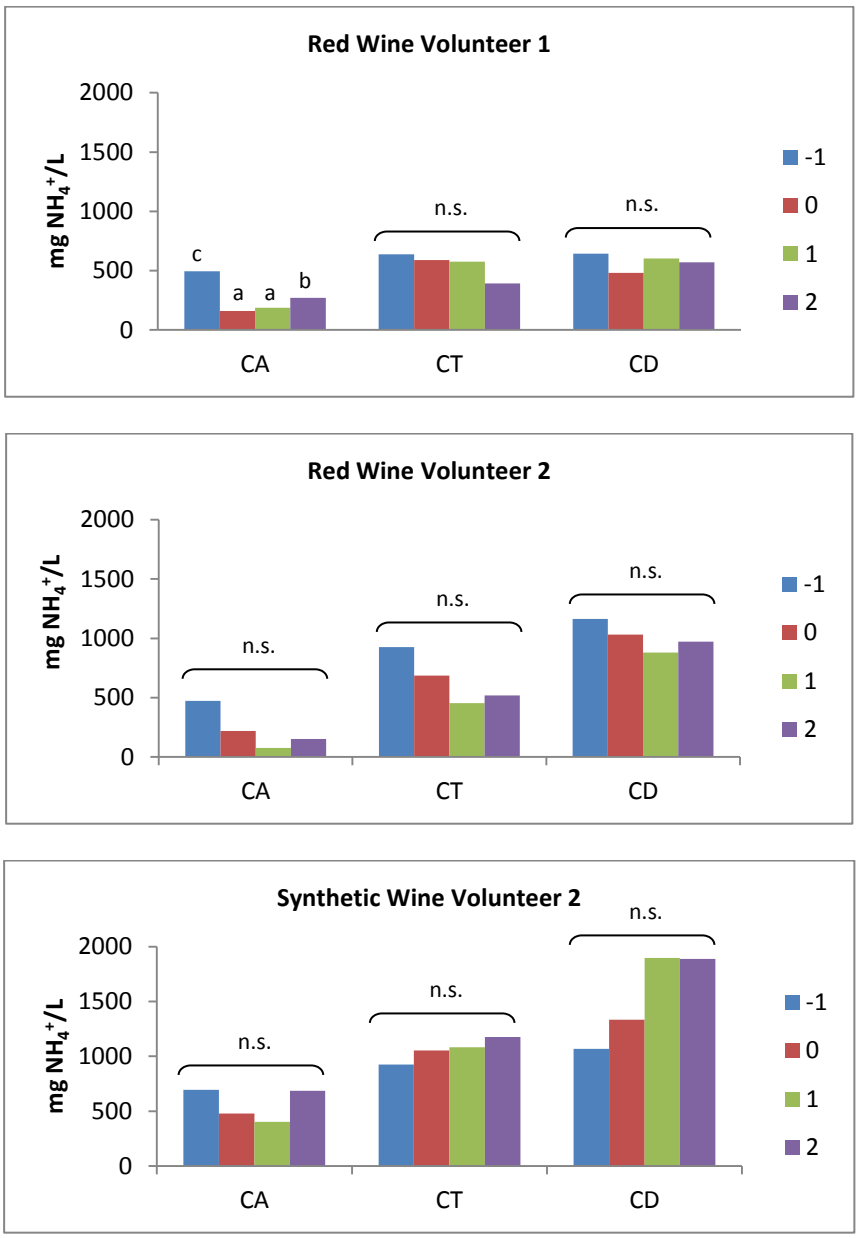
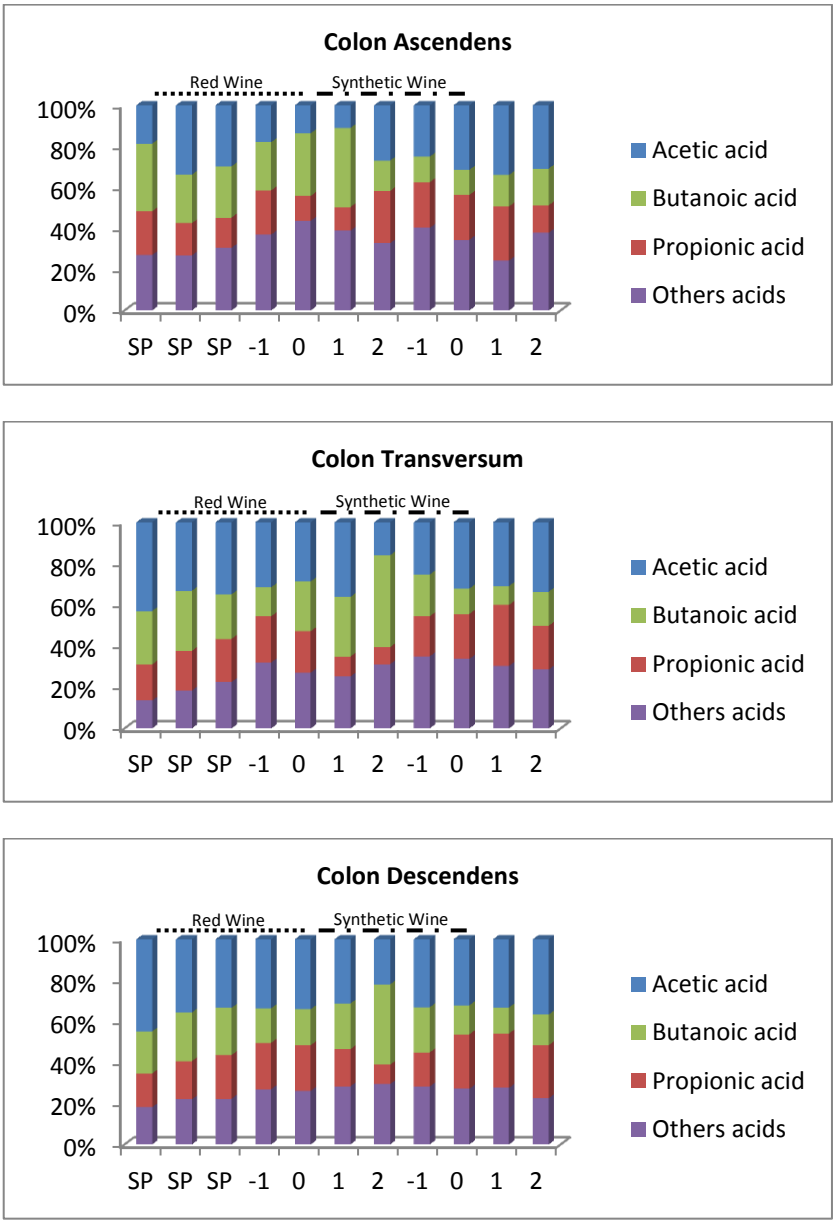

Red Wine Volunteer 1
mg NH_4^+/L
2000
1500
1000
500
0
c
a
a
b
n.s.
n.s.
CA
CT
CD
-1
0
1
2
Red Wine Volunteer 2
mg NH_4^+/L
2000
1500
1000
500
0
n.s.
n.s.
n.s.
CA
CT
CD
-1
0
1
2
Synthetic Wine Volunteer 2
mg NH_4^+/L
2000
1500
1000
500
0
n.s.
n.s.
n.s.
CA
CT
CD
-1
0
1
2

Figure 3.


Colon Ascendens
Red Wine
Synthetic Wine
100%
80%
60%
40%
20%
0%
SP SP SP -1 0 1 2 -1 0 1 2
Acetic acid
Butanoic acid
Propionic acid
Others acids
Colon Transversum
Red Wine
Synthetic Wine
100%
80%
60%
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SP SP SP -1 0 1 2 -1 0 1 2
Acetic acid
Butanoic acid
Propionic acid
Others acids
Colon Descendens
Red Wine
Synthetic Wine
100%
80%
60%
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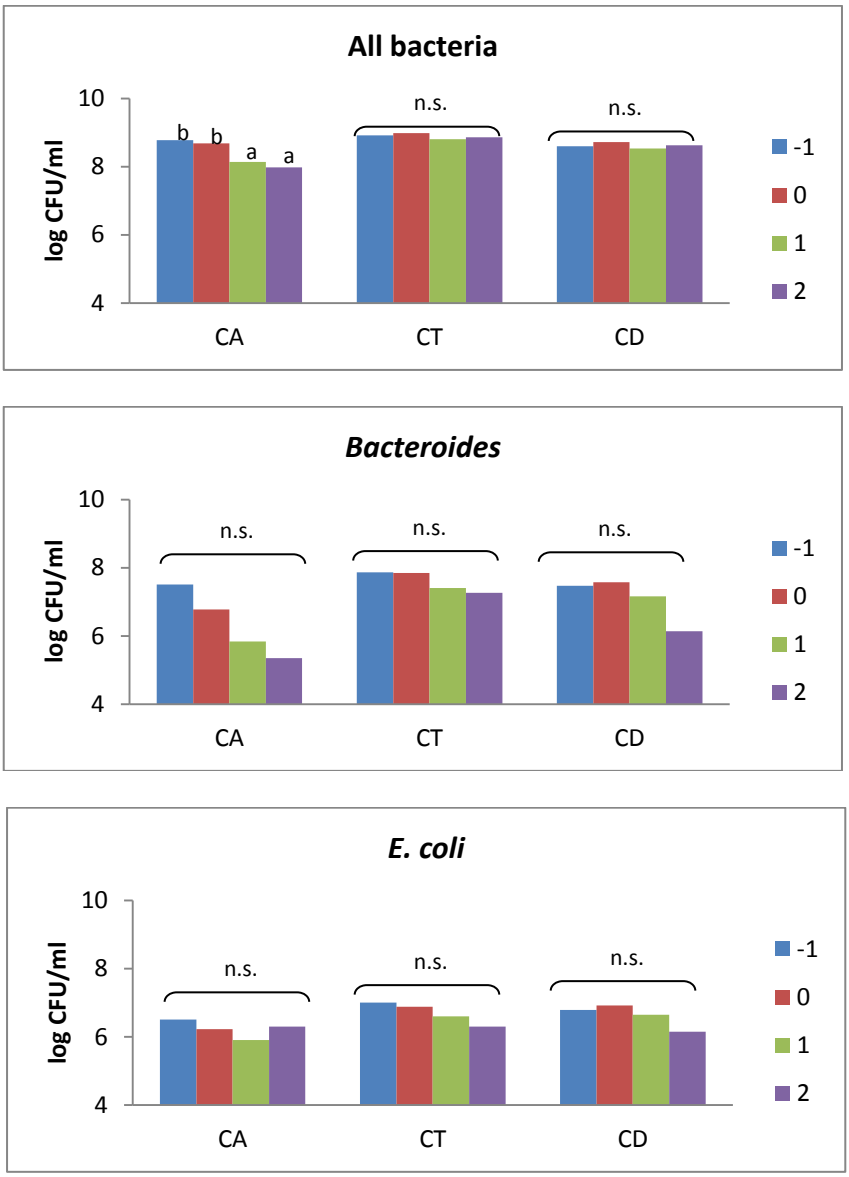
SP SP SP -1 0 1 2 -1 0 1 2
Acetic acid
Butanoic acid
Propionic acid
Others acids

Figure 4.


All bacteria
log CFU/ml
10
8
6
4
b
b
a
a
n.s.
n.s.
CA
CT
CD
-1
0
1
2
Bacteroides
log CFU/ml
10
8
6
4
n.s.
n.s.
n.s.
CA
CT
CD
-1
0
1
2
E. coli
log CFU/ml
10
8
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4
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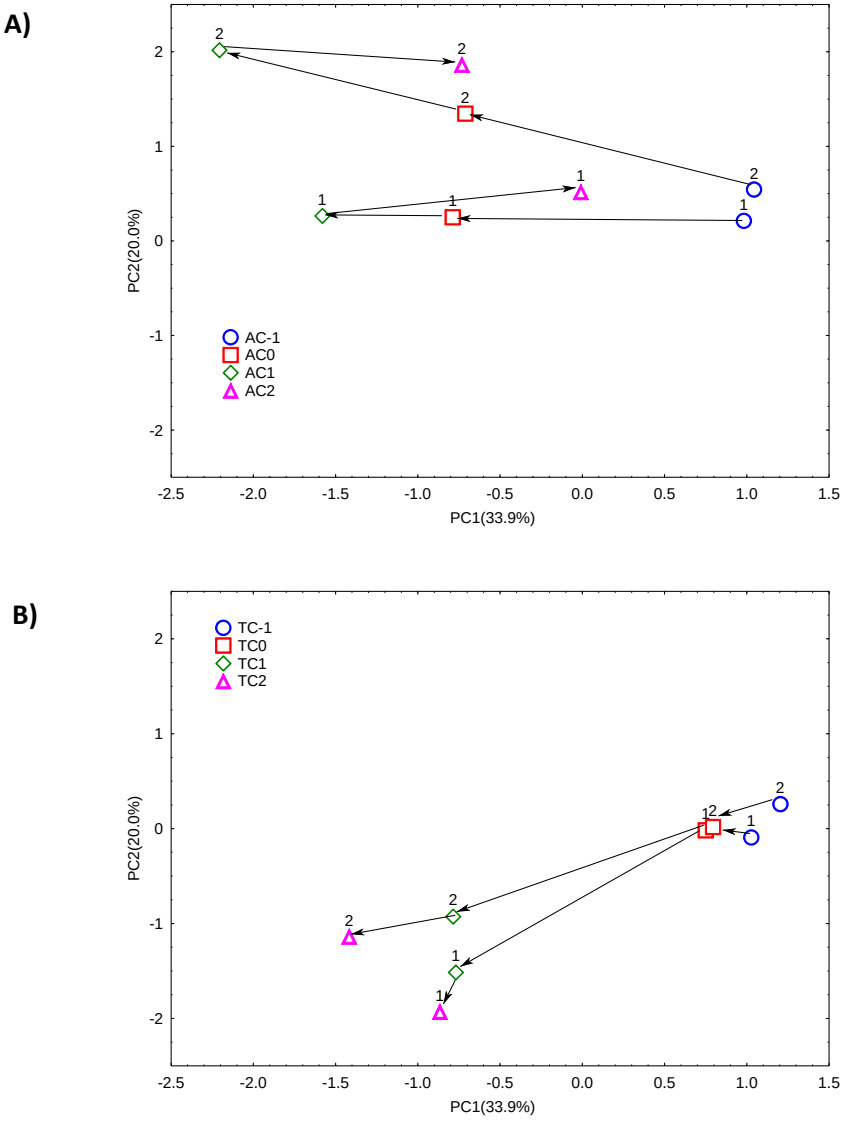
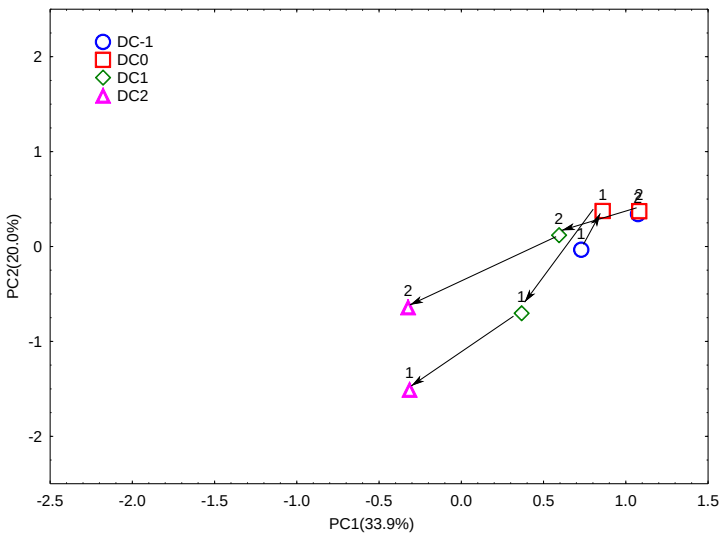
-1
0
1
2

Figure 5.


A)
PC2(20.0%)
PC1(33.9%)
AC-1
AC0
AC1
AC2
B)
TC-1
TC0
TC1
TC2
PC2(20.0%)
PC1(33.9%)

C)



5.3. Simulación de la ingesta de vino tinto mediante modelos *in vitro*

Los estudios *in vitro,* además de complementar las conclusiones globales que se pueden obtener mediante condiciones *in vivo*, se muestran como una alternativa fiable y fácilmente controlable para evaluar de forma simplificada los procesos que tienen lugar en relación a la función de un alimento en el mantenimiento de la salud así como relacionar los componentes de la dieta con diferentes patologías. Dada la dificultad de establecer una relación directa entre la actividad biológica *in vitro* y el efecto *in vivo*, la realización complementaria de ambos permite tener una visión en conjunto para avanzar en el conocimiento de cómo el vino y los polifenoles del vino son metabolizados por la microbiota humana y, a su vez, cómo la microbiota puede verse modulada, por los compuestos y metabolitos fenólicos generados. Por tanto, en este capítulo se han llevado a cabo dos estudios *in vitro*, que engloban el empleo de dos modelos diferentes de simulación, tanto de las condiciones de la placa dental supragingival (apartado 5.3.1) como del tracto gastrointestinal humano (5.3.2), con el objetivo integral de obtener el máximo grado de evidencia científica a nivel de las consecuencias biológicas (y de los mecanismos de acción), derivadas del efecto de la ingesta moderada de vino a nivel de la función digestiva.

5.3.1. Efecto antimicrobiano del vino y extractos enológicos en un modelo de biopelícula oral

La cavidad oral de los individuos sanos contiene cientos de especies bacterianas, virales, y de hongos diferentes, microorganismos que se encuentran asociadas comúnmente en forma de biopelícula oral o "placa dental". La placa dental consiste en la acumulación heterogénea de bacterias aerobias y anaerobias, conviviendo de forma simbiótica, y rodeadas por una matriz intercelular de polímeros de origen salival y microbiano, que se adhiere al esmalte de los dientes o al espacio gingival dentario (Marsh, 2004). Esta asociación en forma de "placa dental" permite una mayor supervivencia y resistencia a tratamientos antimicrobianos (Hoyle y col. 1991). Por tanto, los modelos de biopelícula oral que simulan la placa dental, conducen a resultados más extrapolables que los realizados con cultivos puros, y han sido ampliamente utilizados para evaluar la eficacia de compuestos/mezclas antimicrobianas. Sin embargo, en la actualidad apenas existen estudios acerca de las propiedades antimicrobianas del vino y/o de sus componentes (i.e. polifenoles) sobre bacterias de la cavidad oral asociadas en forma de biopelícula. Por tanto, el objetivo de este apartado ha sido evaluar el potencial

efecto antimicrobiano del vino tinto de estudio y de varios extractos enológicos, ricos en distintos componentes característicos de la matriz vínica, en un modelo de biopelícula oral que simula la composición bacteriana de placa dental supragingival humana, comprobando a su vez el metabolismo oral de determinados compuestos fenólicos inherentes al vino (**Publicación 5.3.1**.).

<u>Publicación 5.3.1</u> El vino tinto y extractos enológicos ejercen efectos antimicrobianos sobre las bacterias orales de un modelo de biopelícula oral

Red Wine and Oenological Extracts Display Antimicrobial Effects in an Oral Bacteria Biofilm Model

Irene Muñoz-González, Thomas Thurnheer, Pedro J. Martín-Álvarez, Begoña Bartolomé, M. Victoria Moreno-Arribas. *Journal of Agricultural and Food Chemistry*, **2014,** 62:4731-4737

Resumen:

Se han estudiado los efectos antimicrobianos del vino tinto y sus componentes inherentes sobre la microbiota oral, mediante el uso de un modelo de biopelícula de la placa supragingival que incluye 5 especies bacterianas: *Actinomyces oris*, *Fusobacterium nucleatum*, *Streptococcus oralis*, *Streptococcus mutans* y *Veillonella dispar*, comúnmente asociadas al desarrollo de la caries dental, cuya complicación puede derivar en el desarrollo de enfermedades orales más graves como la periodontitis o gingivitis. Se estudiaron varias soluciones: un vino tinto rico en polifenoles, el mismo vino tinto desalcoholizado y un extracto fenólico de vino (Provinols™), especialmente rico en antocianinas, el cual se enriqueció bien con un extracto de semilla de uva (Vitaflavan®) rico en flavan-3-oles, o bien con extractos de levaduras secas inactivas (IDY) ricos en manoproteínas o péptidos. El análisis microbiológico (recuento de unidades formadoras de colonias (UFC) y microscopía confocal láser de barrido (CLSM)) de los biofilm tras la aplicación de las diferentes soluciones mostró que la solución de extracto de semilla de uva fue la más efectiva, presentando una alta actividad antimicrobiana frente a *F. nucleatum*, *S. oralis* y *A. oris*. Además, tanto el vino tinto como el vino tinto desalcoholizado mostraron un efecto antimicrobiano frente a *F. nucleatum* y *S. oralis*. También se evaluó el metabolismo de los polifenoles del vino por parte de las bacterias del biofilm, comprobándose la degradación de los precursores de flavan-3-oles, como la (+)-catequina o la procianidina B2, cuando se incubaron los biofilm con el extracto de vino, que no se detectó cuando éstos fueron incubados con el extracto de semilla de uva, probablemente debido a la mayor concentración de flavan-3-oles y, por consiguiente, a su efecto antimicrobiano. En nuestro conocimiento, este es el primer estudio acerca de las propiedades antimicrobianas de vino en un modelo biofilm de la placa dental.

Este trabajo ha sido seleccionado por la American Chemical Society (ACS) como 'topic' para su publicación en el espacio semanal del '**ACS News Service Weekly PressPac**' con el título '*Not just for the heart, red wine shows promise as cavity fighter*' (May 21, 2014):

- http://www.acs.org/content/acs/en/pressroom/presspacs/2014/acs-presspac-may-21-2014/red-wine-shows-promise-as-cavity-fighter.html

Además, el trabajo ha generado un gran interés y ha tenido una gran difusión tanto a nivel nacional como internacional. A continuación se muestra un breve resumen sobre de la difusión del trabajo:

Nivel nacional	**Nivel internacional (Inglés, francés e italiano)**
• 1 periódico nacional (tirada digital) • 1 revista de divulgación médica • 1 revista de divulgación sobre enología • 1 revista de divulgación sobre alimentación/nutrición • 2 revistas de divulgación científica • 2 revistas tipo Magazine • 1 radio	• 12 periódicos (tirada digital) • 16 revista de divulgación médica/salud • 1 revista de divulgación sobre enología • 1 revista de divulgación sobre alimentación/nutrición • 2 revistas de divulgación científica • 4 revistas tipo Magazine • 1 radio

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Red Wine and Oenological Extracts Display Antimicrobial Effects in an Oral Bacteria Biofilm Model

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ABSTRACT: The antimicrobial effects of red wine and its inherent components on oral microbiota were studied by using a 5-species biofilm model of the supragingival plaque that includes *Actinomyces oris*, *Fusobacterium nucleatum*, *Streptococcus oralis*, *Streptococcus mutans* and *Veillonella dispar*. Microbiological analysis (CFU counting and confocal laser scanning microscopy) of the biofilms after the application of red wine, dealcoholized red wine, and red wine extract solutions spiked or not with grape seed and inactive dry yeast extracts showed that the solutions spiked with seed extract were effective against *F. nucleatum*, *S. oralis* and *A. oris*. Also, red wine and dealcoholized wine had an antimicrobial effect against *F. nucleatum* and *S. oralis*. Additional experiments showed almost complete and early degradation of flavan-3-ol precursors [(+)-catechin and procyanidin B2] when incubating biofilms with the red wine extract. To our knowledge, this is the first study of antimicrobial properties of wine in an oral biofilm model.

KEYWORDS: *wine, polyphenols, oral bacteria biofilm, antimicrobials*

INTRODUCTION

The oral cavity is an enormously complex habitat with several hundred commensal microbial species colonizing it, and furthermore, it is unique in the human body in possessing nonshedding surfaces, the teeth, allowing microorganisms to adhere to the surface of teeth for long periods of time, embedded in a self-produced matrix of extracellular polymeric substances,[1] and thus leading to extensive biofilm formation, dental plaque,[2] which is more resistant than planktonic cells to mechanical stress or antibiotic treatment.[3] The microorganisms of dental plaque live with one another in a commensal or mutualistic symbiotic relationship, allowing a mixture of aerobic and anaerobic bacteria to live in the same environment. Some of these oral bacteria, such as streptococci or lactobacilli, are able to produce high levels of organic acids following fermentation of dietary sugars. Acids released from dental plaque lead to demineralization of the tooth surface and consequently to dental caries, periodontal disease or tooth loss,[4] which are the most prevalent oral diseases in humans, affecting up to 60−90% of the world population.[5]

Even using mechanical removal, dental biofilms cannot be eliminated completely. Antimicrobial agents are complementarily used to control dental plaque.[6−8] Until now, several substances have been tested for the control of oral biofilms, including essential oils, amine fluoride, triclosan, etc., but one of the most widely used and effective antibiofilm agents is chlorhexidine.[9] However, chlorhexidine has been associated with some secondary effects, namely the reduction of human taste perception and the pigmentation of oral tissues, which limits its application. Therefore, the search for new antimicrobials has arisen, and natural products are preferable due to the lack of secondary effects and, therefore, the potential for long-term usage in the oral cavity.

The inherent matrix of the biofilm, such as extracellular polymeric substances that reduce penetration of antimicrobial agents and the presence of persistent cells surviving at low metabolic rates, contributes to the widely described phenomenon of reduced sensitivity to antimicrobial agents.[10] Because of this, biofilm models including bacteria and fungi from different species, have proven both useful and reliable in predicting *in vivo* efficacy of antimicrobials. In this sense, most experimental models for short-term studies involve a solid surface for the adhesion of bacteria.[11]

Although there is substantial literature reporting the antimicrobial properties of phenolic compounds or polyphenols against bacteria isolates,[12−14] information about their effect on oral pathogens is still scarce.[15] Studies carried out with tea and cranberry polyphenols have shown an inhibitory effect on biofilm formation by oral pathogens such as *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus sobrinus* and *Porphyromonas gingivalis*.[16] Grapes and wines are good dietary sources of polyphenolic compounds, including hydroxybenzoic and hydroxycinnamic acids, phenolic alcohols, flavan-3-ol monomers, oligomeric and polymeric procyanidins, flavonols, stilbenes and anthocyanins (only present in red varieties).[17] Recently, it has been found that wine and grape phenolic extracts, as well as pomace phenolic extracts, were able to inhibit the growth of different *Streptococcus* spp. strains associated with dental caries.[18,19]

On the other hand, interactions between wine phenolics and oral microbiota can also include a possible bacterial catabolism

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of wine phenolics into less complex phenolic metabolite structures, as seems to happen with flavonol glycosides.[15] With regard to anthocyanins, their degradation in human saliva at 37 °C has been described, being structure-dependent, largely mediated by oral microbiota, and partially suppressed after oral rinsing with antibacterial chlorhexidine.[8]

With the final aim of seeking natural products that could be used in oral hygiene and to ascertain interactions between wine components and oral microbiota, in this study the antimicrobial effects of red wine and dealcoholized red wine were investigated using a biofilm model of the supragingival plaque that integrates five bacteria species commonly associated with oral disease. A wine phenolic extract (Provinols), especially rich in anthocyanins, was also tested using the same model, and in both the absence and presence of other enological extracts from grape seeds (Vitaflavan) and yeast (inactive dry yeast, IDY). Additional experiments were carried out to determine any possible phenolic metabolism during the formation of the bacterial biofilm.

MATERIALS AND METHODS

Red Wines. The red wine used in this study was a young red wine (var. Pinot Noir, vintage 2010), kindly provided by Bodegas Miguel Torres S.A. (Catalonia, Spain). The wine was elaborated following the winery's own winemaking procedures and was selected because of its relatively high phenolic content: total polyphenols = 1758 mg of gallic acid equiv/L, total anthocyanins = 447 mg of malvidin-3-glucoside/L, and total catechins = 1612 mg of (+)-catechin/L. The main individual phenolic compounds found in this wine included anthocyanins, flavan-3-ols, flavonols, alcohols, stilbenes and hydroxycinnamic acids[20] (Table 1).

For the preparation of dealcoholized red wine, ethanol was removed using a rotary evaporator and then distilled water was added until the original volume was reached.

Oenological Extracts. A wine extract, Provinols, was kindly supplied by Safic-Alcan Especialidades S.A.U. (Barcelona, Spain). A grape seed extract, Vitaflavan, was kindly provided by Piriou (Les Dérivés Resiniques & Terpéniques S.A., France). The total phenolic content of the extracts was 474 mg of gallic acid equiv/g for Provinols and 629 mg of gallic acid equiv/g for Vitaflavan. The main phenolic compounds identified in both extracts are reported in Table 1. Also, two inactive dry yeast (IDY) commercial preparations (*Saccharomyces cerevisiae*), IDY 1 and IDY 2, rich in mannoproteins, amino acids and peptides, respectively, were kindly provided by Lallemand S.A. (Blagnac, France) and Agrovin S.A. (Alcázar de San Juan, Ciudad Real, Spain).

The wine extract was dissolved in distilled water containing 2.5% DMSO (v/v), at a concentration of 1.6 g/L. The wine extract solution was fortified in grape seed polyphenols by adding 2.5 g of grape seed extract to 100 mL of the wine solution. Also, the wine extract solution was enriched in wine matrix components (mainly polysaccharides and nitrogen compounds) by adding the IDY preparations to the wine extract solution at a final concentration of 0.4 g/L.

Bacterial Strains and Culture (Growth) Conditions. *Actinomyces oris* OMZ 745, *Fusobacterium nucleatum* OMZ 598, *Streptococcus oralis* OMZ 607, *Streptococcus mutans* UA159 (OMZ 918) and *Veillonella dispar* ATCC 17748^T (OMZ 493) were obtained from the culture collection of the Institute of Oral Biology, University of Zürich. Prior to the experiment, precultures were prepared by transferring the strains on Columbia Blood Agar plates and incubating them for 96 h at 37 °C under anaerobic conditions. After this time, the strains were transferred from the Columbia Blood Agar plates to broth cultures (1 × 9 mL of modified fluid universal medium (mFUM) + 0.3% glucose) (OMZ 493 + 1% sodium lactate) and incubated overnight at 37 °C. After incubation, 200 μL of bacteria from each working culture was individually inoculated in 5 mL of fresh mFUM and incubated at 37 °C anaerobically (7 h maximum). In order to obtain an inoculum

Table 1. Main Phenolic Compounds in Wine and Extracts

	wine (mg/L)[20]	wine extract (mg/g)[34]	grape seed extract (mg/g)[35]
	Benzoic Acids		
Gallic acid	27.30 ± 0.20	1.06 ± 0.05	9.11 ± 0.01
Protocatechuic acid	3.88 ± 0.01	*n.a*[a]	*n.a*[a]
3-*O*-Methylgallic acid	1.06 ± 0.06	*n.a*[a]	*n.a*[a]
4-Hydroxybenzoic acid	0.57 ± 0.01	*n.a*[a]	*n.a*[a]
Vanillic acid	1.85 ± 0.03	*n.a*[a]	*n.a*[a]
Syringic acid	2.30 ± 0.13	*n.a*[a]	*n.a*[a]
Benzoic acid	1.14 ± 0.06	*n.a*[a]	*n.a*[a]
Salicylic acid	0.21 ± 0.01	*n.a*[a]	*n.a*[a]
	Phenols		
Phloroglucinol	0.33 ± 0.03	*n.a*[a].	*n.a*[a]
Tyrosol	31.40 ± 1.40	18.90 ± 1.30	*n.a*[a]
Dihidroxyphenyl propan-2-ol	0.30 ± 0.04	*n.a*[a]	*n.a*[a]
	Cinnamic Acids		
Caffeic acid	6.97 ± 0.26	*n.a*[a].	*n.a*[a]
p-Coumaric acid	1.39 ± 0.02	*n.a*[a].	*n.a*[a]
Ferulic acid	0.22 ± 0.02	*n.a*[a].	*n.a*[a]
Coutaric acid	8.64 ± 0.01	2.00 ± 0.12	*n.a*[a]
Caftaric acid	4.98 ± 0.33	0.19 ± 0.07	*n.a*[a]
	Stilbenes		
Resveratrol	7.12 ± 0.29	0.43 ± 0.02	*n.a*[a]
Resveratrol-3-*O*-glucoside	*n.a*[a]	9.17 ± 0.17	*n.a*[a]
	Flavan-3-ols and Others		
(+)-Catechin	51.60 ± 1.70	9.90 ± 0.32	74.60 ± 0.09
(−)-Epicatechin	34.90 ± 2.90	6.87 ± 0.15	67.70 ± 0.75
(−)-Epicatechin-3-*O*-gallate	*n.a*[a]	0.23 ± 0.02	26.20 ± 0.41
Procyanidin B1	79.10 ± 0.90	11.10 ± 0.10	61.00 ± 1.42
Procyanidin B2	44.70 ± 0.60	4.69 ± 0.10	45.10 ± 0.95
B2-3-*O*-gallate	*n.a*[a]	0.03 ± 0.01	1.80 ± 0.06
B2-3′-*O*-gallate	*n.a*[a]	0.03 ± 0.00	1.61 ± 0.01
Procyanidin B3	16.00 ± 1.00	1.23 ± 0.02	20.40 ± 0.33
Procyanidin B4	12.90 ± 0.30	0.83 ± 0.02	15.00 ± 0.13
Procyanidin B5	2.67 ± 0.01	*n.a*[a]	*n.a*[a]
Procyanidin B7	5.75 ± 0.15	*n.a*[a].	*n.a*[a]
Procyanidin C1	14.00 ± 0.40	1.07 ± 0.04	7.07 ± 0.08
Other trimers	7.96 ± 1.05	1.24 ± 0.09	6.81 ± 0.06 (t2)
	Flavonols		
Quercetin	1.92 ± 0.01	22.40 ± 0.60	*n.a*[a]
Myricetin	0.70 ± 0.03	2.55 ± 0.07	*n.a*[a]
Kaempherol	*n.d*[b]	0.04 ± 0.01	*n.a*[a]
Quercetin-3-*O*-glucoside	*n.a*[a]	0.14 ± 0.02	*n.a*[a]
Quercetin-3-*O*-galactoside	*n.a*[a]	0.11 ± 0.01	*n.a*[a]
	Anthocyanins		
Delphinidin-3-*O*-glucoside	2.58 ± 0.11	0.57 ± 0.012	*n.a*[a]
Cyanidin-3-*O*-glucoside	0.76 ± 0.04	0.27 ± 0.01	*n.a*[a]
Petunidin-3-*O*-glucoside	4.06 ± 0.13	1.47 ± 0.03	*n.a*[a]
Peonidin-3-*O*-glucoside	18.90 ± 2.00	1.78 ± 0.01	*n.a*[a]
Malvidin-3-*O*-glucoside	36.70 ± 3.40	9.01 ± 0.50	*n.a*[a]

[a]Not analyzed. [b]Not detected.

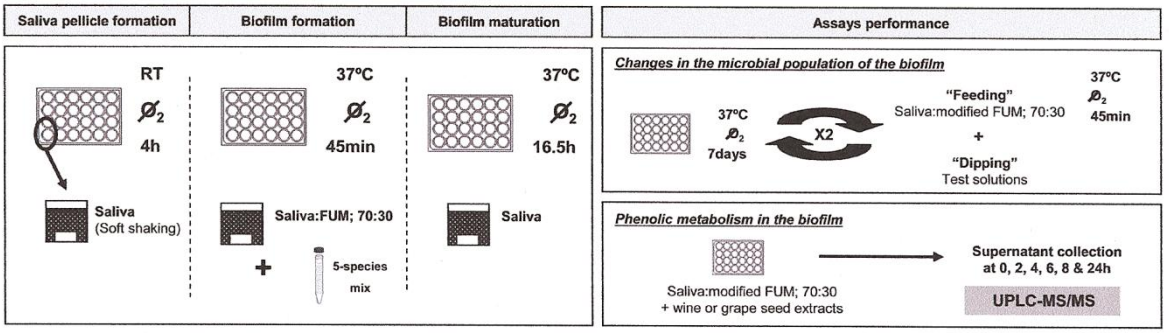

Figure 1. Biofilm formation/maturation and assays diagram.

containing cultures in the exponential growth phase of approximately 10^7 CFU/mL, a microbial suspension with equal volumes and densities of each strain was prepared.

Saliva Processing. Saliva was collected from five volunteers and processed according to the protocol of Guggenheim et al.[21] Briefly, whole unstimulated saliva was collected for 1 h each morning, over several days, at least 1.5 h after eating, drinking, or teeth cleaning. Saliva samples were collected in sterile 50 mL polypropylene tubes, chilled in an ice bath or frozen at −20 °C. After 500 mL of saliva had been collected, it was pooled and centrifuged (30 min, 4 °C, 27,000*g*); the supernatant was pasteurized (60 °C, 30 min) and recentrifuged in sterile tubes. The resulting supernatant was stored in sterile 50 mL polypropylene tubes at −80 °C. The efficiency of the process was assessed by plating the processed saliva samples onto CBA agar; after 72 h at 37 °C, no CFUs were observed on the incubated plates. A sterile 1:1 dilution in H_2O + 25% physiological NaCl was used for the biofilm formation and throughout the experimentation.

In Vitro Biofilm Experiments. Figure 1 shows a sequence chart regarding the biofilm formation prior to assays for determining changes in the microbial population of the biofilm and for assessing phenolic metabolism in the biofilm.

Biofilm Formation. Biofilms were grown using the slightly modified protocol described by Guggenheim et al.[21] and Thurnheer et al.[22] In brief, the 5-species biofilms were grown in 24-well polystyrene cell-culture plates on hydroxyapatite (HA) discs of 9 mm Ø (Clarkson Chromatography Products, South Williamsport, USA) previously preconditioned in 800 μL of whole unstimulated pooled saliva (as described in the previous section) during 4 h at room temperature, with shaking (95 rpm) in order to promote pellicle formation. To initiate the biofilm formation, the discs were covered for 45 min with 1.6 mL of a mixture comprising 30% saliva, 70% mFUM and 200 μL of the bacterial inoculum described above. mFUM corresponds to a well-established tryptone yeast-based broth medium designated as FUM[23] and modified by supplementing 67 mM Sørensen's buffer (final pH 7.2). The carbohydrate concentration in mFUM was 0.3% (w/v) and consisted of glucose for the first 16 h and from then on of a 1:1 (w/w) mixture of glucose and sucrose.

After this first incubation, discs were subjected to three consecutive 1 min dip-washes in 2 mL 0.9% NaCl to remove growth medium and free-floating cells but not microorganisms adhering firmly to the HA discs. Then, they were incubated anaerobically for 16.5 h at 37 °C in preconditioned and processed saliva to form the biofilm (Figure 1).

Assay for Determining Changes in the Microbial Population of the Biofilm. Once the biofilm was formed, discs were maintained in a 24-well plate with preconditioned and processed saliva in anaerobic conditions for 7 days. Twice a day, and with 7 h of difference in between, discs were "fed" by immersing them into a preconditioned fresh growth medium (30% saliva, 70% mFUM (v/v) containing 0.15% glucose and 0.15% sucrose) for 45 min, at 37 °C, under anaerobic conditions, After each "feeding", discs were dipped in the different test solutions (1 mL) for 2 min and while being gently shaken by hand. After this time, the discs were dipped once in the preconditioned-processed saliva in order to clean any remains of the test solutions. Immediately after, discs were returned to the "old" 24-well plate with preconditioned and processed saliva and incubated anaerobically until the next "feeding" (Figure 1). After 7 days, biofilms were either stained for confocal laser scanning microscopy (see below) or harvested, at room temperature, in 1 mL of 0.9% NaCl by scratching with a special odontological instrument. Cell viability was tested using a Live/Dead BacLight Viability Kit (Molecular Probes Inc.) The total CFU, streptococci and all taxa were assessed by anaerobic culture (37 °C) using selective (Mitis Salivarious for *Streptococcus oralis* and *Streptococcus mutans*; Fastidious Anaerobe Agar for *Fusobacterium nucleatum*) and nonselective media (Columbia Blood Agar for *Actinomyces oris*, *Veillonella dispar* and total CFU) and colonies were counted.

Distilled water was used as the negative antimicrobial control, and 0.2% chlorhexidine-gluconate solution (Sigma-Aldrich, Steinheim, Germany) in water was the positive antimicrobial control. In order to discard a possible antimicrobial effect of the alcohol, a 12%-ethanol-in-water solution was also tested. For both, test solutions and controls, experiments were carried out in triplicate.

Assay for Assessing Phenolic Metabolism in the Biofilm. After initiating biofilm formation as described above, the 70:30 saliva:mFUM media was enriched with the wine extract (1.6 g/L) in the absence of the presence of grape seed extract (10 g/L) and added into the wells containing the discs (Figure 1). Then, plates were incubated at 37 °C under anaerobic conditions and aliquots of enriched media were taken at 0, 2, 4, 6, 8, and 24 h.

Analysis of Wine Compounds and Bacterial/Microbial Metabolites. Phenolic compounds were analyzed using an UPLC-ESI-MS/MS following a previously reported method.[20] The liquid chromatographic system was a Waters Acquity UPLC (Milford, MA) equipped with a binary pump, an autosampler thermostated at 10 °C, and a heated column compartment (40 °C). The column employed was a BEH-C18, 2.1 × 100 mm and 1.7 μm particle size from Waters (Milford, MA). The mobile phases were 2% acetic acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient program was as follows: 0 min, 0.1% B; 1.5 min, 0.1% B; 11.17 min, 16.3% B; 11.5 min, 18.4% B; 14 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B. Equilibrium time was 2.4 min resulting in a total runtime of 18 min. The flow rate was set constant at 0.5 mL/min and injection volume was 2 μL.

The LC effluent was pumped to an Acquity TQD tandem quadrupole mass spectrometer equipped with a Z-spray electrospray ionization (ESI) source operated in negative polarity mode. The ESI parameters were set as follows: capillary voltage, 3 kV; source temperature, 130 °C; desolvation temperature, 400 °C; desolvation gas (N_2) flow rate, 750 L/h; cone gas (N_2) flow rate, 60 L/h. The ESI was operated in negative ionization mode. For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific to each compound. The MS/MS parameters (cone voltage, collision energy and MRM transition) of the 60 phenolic compounds targeted in the present study (mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, 4-hydrox-

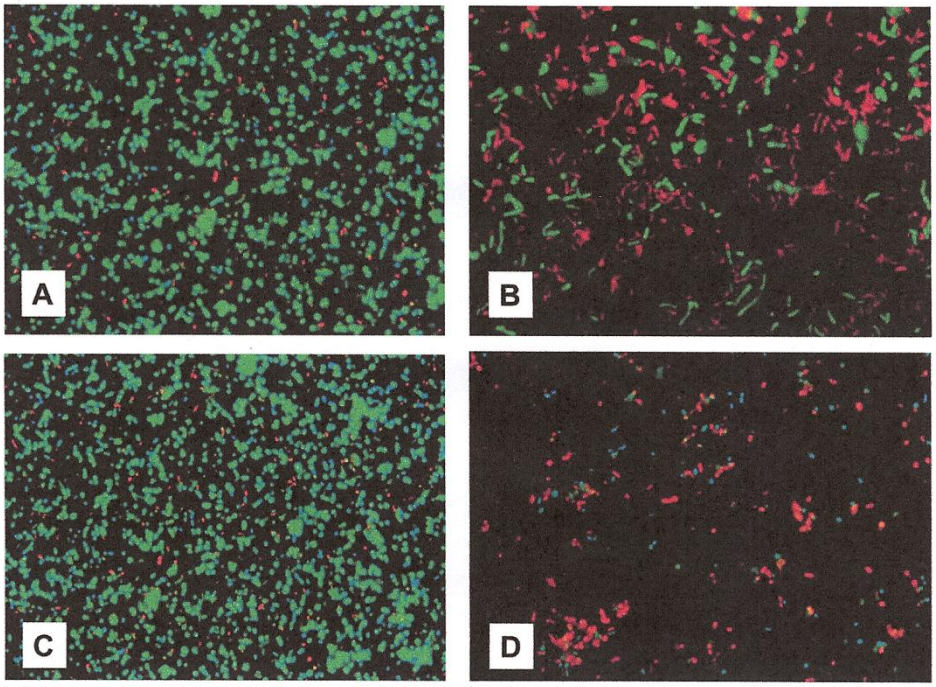

Figure 2. Confocal scanned segment of biofilm stained with LIVE/DEAD kit after exposure to (A) negative control (water), (B) red wine, (C) wine extract (Provinols, 1.6 g/L), and (D) grape seed extract (Vitaflavan, 2.5 g/L) in wine extract solution (1.6 g/L).

Table 2. Bacterial Populations (Log_{10}CFU) of *S. mutans*, *S. oralis*, *A.oris*, *F. nucleatum*, and *V. dispar* in the Biofilm, after Treatments with Water, Ethanol 12% in Water, Wine, Dealcoholized Wine and 0.2% Clorhexidine-gluconate

	S. mutans	*S. oralis*	*F. nucleatum*	*A. oris*	*V. dispar*
Water	8.09 ± 0.09	8.42 ± 0.17	5.90 ± 0.89	8.40 ± 0.32	7.36 ± 0.37
Ethanol 12% in water	8.01 ± 0.16	8.20 ± 0.37	<1.30 ± 0.00[a]	8.75 ± 0.64	7.92 ± 0.12
Wine	7.89 ± 0.07	5.77 ± 0.63[a]	<1.30 ± 0.00[a]	8.37 ± 0.20	6.94 ± 0.38
Dealcoholized wine	7.68 ± 0.22	4.79 ± 0.80[a]	<1.30 ± 0.00[a]	8.24 ± 0.07	7.12 ± 0.88
Clorhexidine-gluconate 0.2%	<1.30 ± 0.00[a]	<1.30 ± 0.00[a]	<1.30 ± 0.00[a]	<1.30 ± 0.00[a]	<1.30 ± 0.00[a]

[a]Significant differences (Dunnett's test) in the population in comparison to the negative control (water).

yvaleric acids and valerolactones) were previously reported.[24] Data acquisition and processing was realized with MassLynx 4.1 software.

Staining of Biofilms and Confocal Laser Scanning Microscopy (CLSM). For CLSM, treated as well as untreated, biofilms were stained using the LIVE/DEAD BacLight bacterial viability assay (Invitrogen, Zug, Switzerland) according to the instructions of the manufacturer. After 20 min staining, excess dye was gently aspirated from the discs without touching the biofilms. They were embedded upside-down in 20 μL of Mowiol[25] and stored at room temperature in the dark for at least 6 h prior to microscopic examination.

Stained biofilms were examined by CLSM at randomly selected positions using a Leica TCS SP5 (Leica Microsystems, Heidelberg GmbH, Germany) with a ×20/0.8 numerical aperture (NA) and a ×63/1.4 NA oil immersion objective lens in conjunction with 488 nm laser excitation and 530 nm emission filters for Syto 9 (live stain) and 561 nm laser excitation, and 640 nm emission filters for propidium iodide (dead stain). Image acquisition was done in 8-line average mode, and the data were processed using Imaris 7.2.2 (Bitplane AG, Zurich, Switzerland).

Statistical Analysis. Means and standard deviations were calculated using Microsoft Excel 2007. Statistical analyses were performed through Statistica©. To compare the antimicrobial effects of the different treatments with the control (water), the Dunnett test was applied. Graphs were performed with Microsoft Excel 2007.

RESULTS AND DISCUSSION

Antimicrobial Properties of Red Wine on the Biofilm. The effects of a red wine and the same wine without ethanol on a biofilm model composed of five representative species commonly encountered in supragingival plaque, including Gram-positive (*A. oris, S. mutans, S. oralis*) as well as Gram-negative (*F. nucleatum, V. dispar*) bacteria,[21] were investigated. Among these bacteria were the so-called early colonizers, *A. oris, S. oralis* and *V. dispar*, and late colonizers, *S. mutans* and *F. nucleatum*, the latter also designated as a bridging organism due to its capability to coaggregate with a wide range of early and late colonizers.[26] In comparison to the control biofilm (Figure 2A), when biofilms were dipped into red wine (Figure 2B) and dealcoholized red wine, some decrease in cell viability of the whole biofilm was visually estimated. However, no visually changes were observed when applying the wine extract solution (Figure 2C). CFU values for the five bacteria comprising the biofilm indicated an important reduction in *F. nucleatum* and *S. oralis* population when applying red wine and dealcoholized red wine to the biofilm, in comparison to the negative control (distilled water) (Table 2). The Dunnett test confirmed

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Table 3. Bacterial Populations (Log_{10}CFU) of *S. mutans*, *S. oralis*, *A.oris*, *F. nucleatum*, and *V. dispar* in the Biofilm, after Treatments with Provinols, Provinols + Vitaflavan, Provinols + IDY 1 and Provinols + IDY 2

	S. mutans	*S. oralis*	*F. nucleatum*	*A. oris*	*V. dispar*
Water + 2.5% DMSO	8.02 ± 0.05	8.47 ± 0.09	6.77 ± 0.07	8.55 ± 0.07	7.74 ± 0.04
Provinols	8.11 ± 0.08	8.59 ± 0.11	6.54 ± 0.57	8.34 ± 0.39	7.68 ± 0.39
Provinols + Vitaflavan	7.77 ± 0.18	6.49 ± 0.07[a]	<1.30 ± 0.00[a]	<3.30 ± 0.00[a]	7.95 ± 0.09
Provinol + IDY 1	8.18 ± 0.03	8.60 ± 0.01	7.13 ± 0.13	8.89 ± 0.02	8.15 ± 0.15
Provinols + IDY 2	8.13 ± 0.07	8.44 ± 0.07	7.14 ± 0.04	8.68 ± 0.03	8.11 ± 0.11

[a]Significant differences (Dunnetts test) in the population in comparison to the negative control (water + 2.5% DMSO).

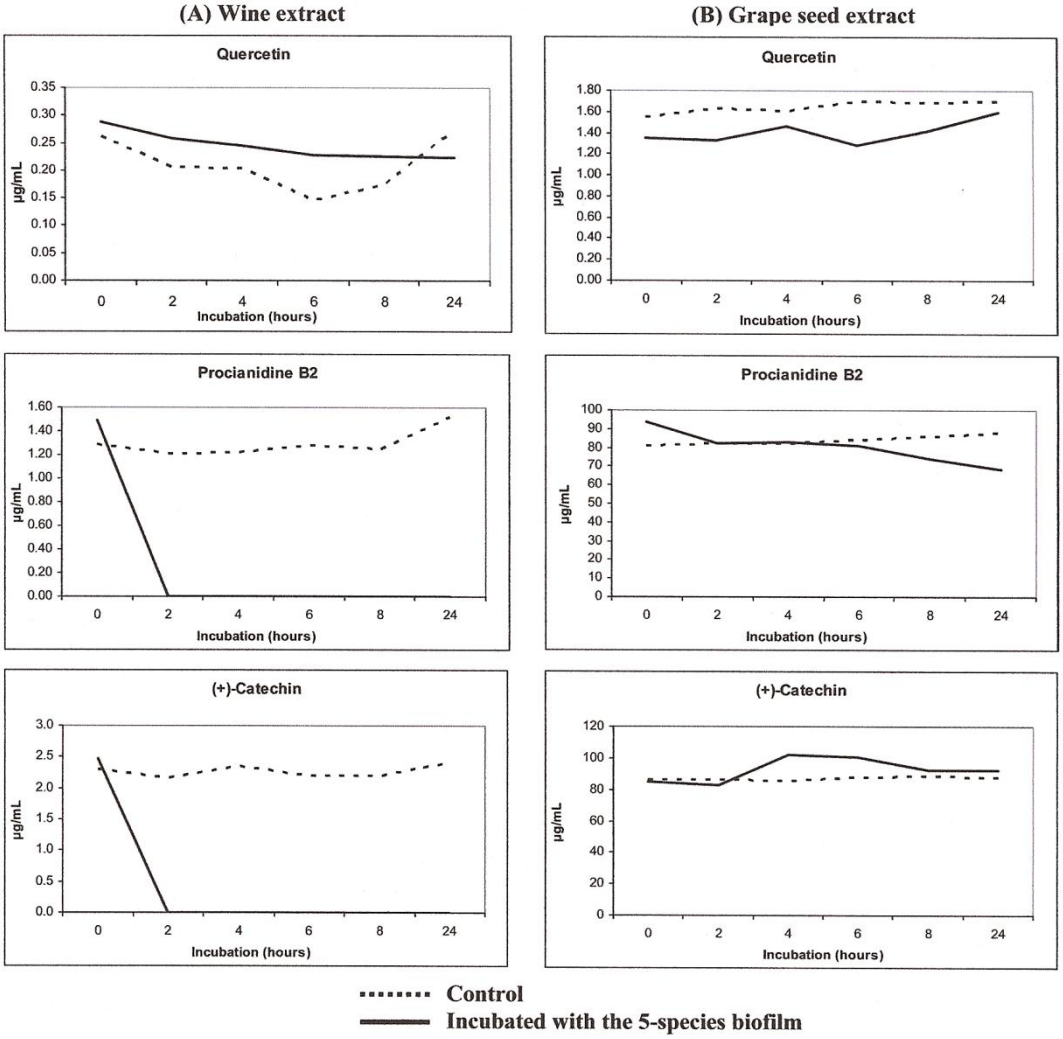

Figure 3. Metabolism of precursors (+)-catechin, quercetin and procyanidin B2 after 0, 2, 4, 6, 8, and 24 h of incubation in FUM media enriched with (A) wine extract (Provinols) and (B) grape seed extract (Vitaflavan, 1%) in wine extract solution.

significant differences in the population of these two strains after the treatment with wine and dealcoholized wine. Generally, wines contain between 10 and 12% of ethanol, which have antimicrobial properties. To understand the action mechanism of red wines in more depth, the effects of ethanol of the bacteria biofilm were investigated. The treatment with 12% ethanol resulted in a significant decrease in the population of *F. nucleatum* (Table 2). However, since treatments of the biofilm with both wine and dealcoholized wine inhibited *F. nucleatum* growth, it was likely that other wine components—apart from ethanol—had antimicrobial properties against this bacteria species. As expected, all the strains were eradicated after the treatment with the positive control (0.2% chlorexidine-gluconate solution) (Table 2).

In an intervention study with 75 volunteers, Signoretto et al.[27] analyzed the microbial population of supragingival and

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subgingival plaque using PCR-DGGE and found that *F. nucleatum* was less frequent in wine drinkers compared with water drinkers. Other authors, such as Daglia et al.,[28] have also shown antimicrobial properties of dealcoholized wine against oral streptococci. Both studies were consistent with our results in that wine selectively inhibited the growth of *F. nucleatum* and *S. oralis* in the presence of other species, such as *S. mutans*, *A. oris* and *V. dispar* in an oral biofilm model.

Given the antimicrobial effects of wine observed in the first experiment, the next step was to study the influence of some wine-specific components, such as polyphenols, including flavan-3-ols, peptides or yeast polysaccharides. For that purpose, a red wine extract solution spiked with different extracts rich in those specific components of wine (grape seed extract rich in flavan-3-ols, and two inactive dry yeasts rich in peptides and mannoproteins, respectively) was used. Table 3 reports the CFU values of the five bacteria species of the tested biofilm after treatments with wine extract and wine extract solution spiked with different extracts (grape seed extract, IDY1 and IDY2). Dunnett's test showed significant differences in *F. nucleatum, S. oralis and A. oris* with the application of the wine extract spiked with the grape seed extract rich in flavan-3-ols.[17] However, wine extract solutions spiked with IDY1 and IDY2 did not show any effect in the populations of the five-strain biofilm. Notably, a great decrease in the viability of the cell was visually appreciated in the biofilm recovered from the discs that were dipped in the grape seed extract solution (Figure 2D). Cueva et al.[29] reported significant inhibition in the growth of some oral streptococci, such as *Streptococcus mutans* and *Streptococcus sobrinus*, when incubating planktonic cultures with flavan-3-ols precursors, (+)-catechin and (−)-epicatechin, in which grape seed extract is particularly rich. Moreover, they showed that extracts from grape seed, especially Vitaflavan and its oligomeric fraction, exerted higher antimicrobial activity against various oral pathogens than the rest of the extracts tested (red wine extract and grape pomace extract). Similarly, Rotava et al.[30] and Baydar et al.[31] reported antimicrobial effects of grape seed extracts against pathogenic bacteria such as *S. aureus* and *E. coli*. It has been suggested that the high concentration of flavonoids and their derivatives in grape seeds could be responsible for the antimicrobial activity of grape seed extracts.[32] These observations raise the question of how the hydroxyl groups (structure) of flavonoids affect oral bacterial biofilm.

The search for new antimicrobial agents to control the formation of dental plaque requires appropriate screening models that include orally relevant organisms. The mode used in this study is not only useful for investigating ecological shifts in plaque composition in response to plaque composition but also for testing the efficacy of antimicrobial agents under conditions of repeated short-term exposure.[11] Other oral biofilm models that use flow chamber systems or in-mouth dispositives have also been used. Among these, flow chamber systems allowing biofilm formation/maturation under hydrodynamic conditions, and careful control and easy changes of the environment, are very useful for analysis of structural biofilm formation. In-mouth dispositives allow *in vivo* formation of the biofilm including the endogenous microbiota of the volunteer. Although these models are the most realistic in miming oral biofilms, the high interindividual variability in the composition of the biofilms may provoke high deviations in the results.

Changes in Wine Phenolic Metabolism. Because wine and its polyphenols diminished bacteria population in the oral biofilm, a new assay was performed in order to gain a deeper understanding about microbial metabolism of polyphenols in the tested extracts.

First, the wine extract solution was added to the growth media and the progress of the phenolic metabolism by the five-species biofilm was studied by monitoring changes in the main phenolic compounds present in the wine extract (Table 1): flavan-3-ols monomers ((+)-catechin, (−)-epicatechin and (−)-epicatechin-3-*O*-gallate), dimeric procyanidins (B1, B2, B3, B4, B5, B7, B2-3-*O*-gallate and B2-3-*O*-gallate), trimeric procyanidins (C1 and other trimers) and flavonols (quercetin, myricetin, kaempherol, quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside). As a brief example, Figure 3A shows the differences in the degradation by the five-strain biofilm of three of the analyzed precursors, (+)-catechin, procyanidin B2 and quercetin, when growing in media enriched with the wine extract solution (1.6 g/L). The UPLC-MS analysis of these three compounds showed high degradation rates, almost completely during the first 2 h of incubation, in the flavan-3-ol precursors, (+)-catechin and procyanidin B2, probably because of their low concentration in the media, which permitted the bacteria of the biofilm to ues them as a carbon source. However, no degradation of the precursor quercetin was observed during the incubation period.

To gain further knowledge about the metabolism of grape polyphenols, specifically in flavan-3-ols metabolism, the growing media was enriched by adding, to the red wine extract solution, a concentration of 10 g/L of grape seed extract, which is especially rich in flavan-3-ol precursors (Figure 3B). Despite the greater concentration of flavan-3-ol precursors, no degradation of the flavan-3-ol precursors was observed. Nor was degradation of precursor quercetin observed during the incubation period.

The emergence of antibiotic resistance by some oral bacteria biofilm species presents a worldwide problem, and thus, novel strategies are required. The use of natural antimicrobials may contribute to controlling the disordered growth of oral microbiota; thus, overcoming problems caused by species resistant to conventional antimicrobials.[33] To our knowledge, this is the first report on the antimicrobial properties of wine in an oral biofilm model. Our results show that red wine, at moderate concentration, inhibits the growth of some pathogenic species in an oral biofilm model. These findings contribute to existing knowledge about the beneficial effects of red wines (one of the most important products of agriculture and food industries) on human health. Moreover, the promising results concerning grape seed extract, which showed the highest antimicrobial activity, open promising ways toward a natural ingredient in the formulation of oral care products specifically indicated for the prevention of caries, due to its antimicrobial properties.

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Notes

The authors declare no competing financial interest.

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5.3.2. Efecto de la ingesta de vino sobre las poblaciones de bacterias intestinales y su metabolismo en un modelo de simulación de tracto gastrointestinal

Los efectos sobre la salud humana asociados a las dietas/alimentos ricos en polifenoles parecen atribuirse, en gran parte, a los metabolitos fenólicos derivados del metabolismo microbiano de los polifenoles en el colon (Requena y col., 2010). Por otro lado, algunos estudios recientes han demostrado que la actividad metabólica de la microbiota del colon en relación a los polifenoles, es región-dependiente (Barroso y col., 2013). Por todo esto, los abordajes experimentales *in vitro* podrían aportar nuevas evidencias científicas para comprobar, de forma más específica, el efecto del vino y sus polifenoles sobre la microbiota colónica y el metabolismo fenólico intestinal. Los modelos de fermentación estática en *batch*, han sido los más utilizados con este fin por su sencillez y bajo coste (Dueñas y col., 2014). Sin embargo, en los últimos años se han desarrollado simuladores dinámicos del tracto gastrointestinal que permiten simular, de forma más aproximada, las condiciones reales y los parámetros fisiológicos que pueden influir en la microbiota y su actividad metabólica (Alminger y col., 2014).

Con este objetivo, en la presente Tesis doctoral, se ha aplicado por primera vez un novedoso simulador *in vitro* del tracto gastrointestinal (SIMGI) para evaluar el efecto de la ingesta moderada de vino tinto sobre la microbiota intestinal y su repercusión en el metabolismo colónico de los polifenoles **(Publicación 5.3.2)**. Para permitir una comparativa con los resultados de experimentación en humanos (Publicación 5.1.2), se ha utilizado el vino tinto de estudio para la alimentación del SIMGI y, para la inoculación de los fermentadores, muestras fecales procedentes de dos individuos involucrados en el citado estudio de intervención y clasificados como de alta y moderada capacidad para metabolizar los polifenoles del vino.

<u>Publicación 5.3.2</u> Aplicación de un nuevo modelo *in vitro* de simulación gastrointestinal (SIMGI) para estudiar el impacto del vino en el metabolismo colónico

Application of a Novel in Vitro Gastrointestinal Simulator (SIMGI) to Study the Impact of Wine in the Colonic Metabolism

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Resumen:

Los polifenoles del vino podrían influir favorablemente en la salud humana mediante la modificación de la actividad metabólica y/o composición de la microbiota intestinal. El objetivo de este estudio fue evaluar el impacto de vino tinto en el metabolismo colónico empleando un Simulador Gastrointestinal *in vitro* (SIMGI), inoculado con muestras fecales de individuos sanos (n=2). En las muestras procedentes de los tres compartimentos del colon –ascendente, trasverso y descendente- se monitorizaron los principales compuestos fenólicos del vino y sus metabolitos, así como el ión amonio, los ácidos grasos de cadena corta (SCFAs) y algunos grupos microbianos, después de la alimentación del sistema con vino tinto (225 mL). Se encontraron aumentos para los ácidos gálico, protocatéquico, 3-O-metilgálico, 4-hidroxibenzoico, 3,4-dihidroxifenilpropiónico, vainillínico, siríngico, y salicílico después de la alimentación puntual con vino. Simultáneamente, se observó una disminución en la concentración de amonio y un aumento de la producción de ácido butírico. Por su parte, la actividad metabólica de la microbiota resultó individuo- y compartimento-dependiente, ocurriendo los principales cambios en el colon ascendente. En resumen, estos resultados destacan que el vino tinto modula la actividad metabólica de la microbiota colonica *in vitro*, lo cual podría ser relevante fisiológicamente. Además, la comparación de estos resultados con los de un estudio anterior de intervención en humanos valida la utilidad de SIMGI como el modelo de simulación gastrointestinal.

DISCUSIÓN GENERAL

6. DISCUSIÓN GENERAL

En los últimos años se ha incrementado el interés acerca de los efectos del consumo moderado de vino sobre la salud humana. Este creciente interés en el binomio VINO-SALUD se debe, por un lado, a que el vino, como alimento de origen natural que forma parte de la dieta mediterránea y cuyo consumo se conoce desde la antigüedad (Willet, 1995) tiene una enorme importancia económica y socio-cultural en España y otros países mediterráneos. Por otro lado, el interés sobre su impacto sobre la salud humana aumentó considerablemente cuando Renaud y Lorgeril (1992) acuñaron el concepto de "paradoja francesa" y atribuyeron al vino un carácter cardioprotector. Desde entonces, son numerosos los estudios epidemiológicos, *in vitro* e *in vivo,* tanto en animales de experimentación como en humanos, con el objetivo de estudiar las propiedades beneficiosas sobre la salud derivadas del consumo moderado de vino, especialmente en relación a la salud cardiovascular (Wollin y col., 2001; Sacanella y col., 2007; Estruch y col., 2011; Aleixandre y col., 2013).

En la última década, el enfoque de los trabajos sobre las propiedades saludables del vino se ha ampliado hacia otros sistemas del organismo humano como los sistemas inmune, nervioso o digestivo. Concretamente, el impacto del consumo moderado de vino del sobre la salud digestiva apenas ha sido investigado y los estudios de intervención/nutricionales en este ámbito son escasos. Para estudiar el comportamiento y los efectos que el vino, como alimento, puede tener sobre la salud digestiva, es necesario llevar a cabo estudios *in vivo* e *in vitro* complementarios que permitan obtener una visión de conjunto acerca del proceso de digestión del vino, y de los mecanismos subyacentes a la relación entre el consumo moderado de vino y la función digestiva.

Enmarcada en este contexto, la presente Tesis Doctoral ha tenido como objetivo evaluar el impacto del consumo moderado de vino sobre la salud digestiva, teniendo en cuenta tanto la perspectiva *in vivo* como la *in vitro*. En una primera parte, se llevó a cabo un estudio de intervención controlado de consumo moderado de vino en un amplio grupo de sujetos sanos, y se evaluó el efecto sobre el metaboloma fecal (publicaciones 5.1.1, 5.1.2 y 5.1.3) y sobre la función intestinal (publicaciones 5.2.1 y 5.2.2). En la segunda parte del trabajo, que engloba la publicación 5.3.1, se buscó conocer las propiedades antimicrobianas de la ingesta de vino tinto sobre bacterias de la microbiota oral en un modelo *in vitro* de biopelícula de la placa supragingival. Finalmente, la tercera parte de la Tesis, que

engloba la publicación 5.3.2, tuvo como principal objetivo profundizar en el metabolismo microbiano de los polifenoles del vino durante su paso por el intestino, empleando para ello un nuevo simulador del tracto gastrointestinal (SIMGI) que reproduce las condiciones dinámicas del intestino humano.

6.1. Estudio de intervención nutricional con vino en humanos: efecto sobre el metaboloma fecal y la función intestinal de sujetos sanos

Se diseñó un estudio de intervención controlado y regular de consumo de vino tinto (250 mL /día) durante un tiempo razonable que permitiera observar cambios a nivel intestinal (4 semanas). Se reclutó un número relativamente alto (n=42) de voluntarios sanos sin antecedentes de enfermedades intestinales. Otro requisito que consideramos importante fue seguir la dieta habitual pero restringida en alimentos ricos en polifenoles, tanto durante el periodo de lavado (2 semanas) como durante la intervención. Teniendo en cuenta que muchas de las propiedades saludables derivadas del consumo moderado de vino han sido atribuidas a su contenido y tipo de compuestos fenólicos o polifenoles, los cuales son ampliamente metabolizados en el colon por la microbiota intestinal dando lugar a metabolitos bioactivos (Monagas, 2010; Williamson y Clifford, 2010), cabe esperar que el análisis de metabolitos fenólicos de origen microbiano en heces permitiera avanzar en el conocimiento de cómo el vino y sus compuestos activos, los polifenoles, actúan sobre el sistema digestivo. Precisamente la consideración de las muestras fecales para la evaluación del efecto de la intervención con vino sobre la función intestinal, es un aspecto original de este trabajo, con respecto a otros previamente publicados en la literatura, que generalmente consideran otros fluidos biológicas, como la orina junto con el plasma y el suero, para llevar a cabo los estudios de metabolitos derivados de alimentos.

Desde el punto de vista analítico, los metabolitos fenólicos originados a partir de los polifenoles por el metabolismo colónico son compuestos de bajo peso molecular y la mayoría están presentes en los fluidos y heces humanas en concentraciones muy bajas, del orden de micromoles (Grün y col., 2008). Por tanto, el análisis de metabolitos fenólicos en heces, dada la complejidad de este tipo de muestra, requiere técnicas analíticas sensibles y robustas que permitan un análisis rápido y fiable. Además, el empleo de técnicas de preparación de la muestra como la extracción en fase sólida (SPE) podría ser

especialmente útil a la hora de analizar muestras fecales, permitiendo reducir el efecto de la matriz por la eliminación de interferencias.

Por ello, la publicación 5.1.1 de la presente tesis doctoral tuvo como objetivo evaluar la idoneidad de la SPE como paso previo al análisis de metabolitos fenólicos en muestras fecales por UPLC-ESI-TQ MS/MS, para lo cual se llevaron a cabo cuatro experimentos diferentes. Nuestros experimentos iniciales nos permitieron seleccionar el relleno Oasis® HLB como el más eficaz en el recuperación de compuestos puros representativos de metabolitos fenólicos fecales (Publicación 5.1.1). Aunque los cartuchos con rellenos apolares han sido ampliamente empleados para la extracción en fase sólida de compuestos fenólicos (Gonthier y col., 2003; Michalkiewicz y col., 2008; Rios y col., 2003), los nuevos rellenos con un balance hidrofílico-lipofílico, como el Oasis® HLB, parecen mejorar la recuperación de determinados compuestos fenólicos, como se ha comprobado previamente en muestras de orina (Medina-Remón y col., 2009; Urpí-Sarda y col., 2009) y en vinos (Baranowska y Magiera, 2011; Perez-Magarino y col. 2008). Una vez seleccionado el cartucho Oasis® HLB, se comprobó que la acidificación previa de la muestra mejoraba notablemente la recuperación de los compuestos fenólicos incluidos en una solución fecal, como también demostraron previamente Choy y col. (2013), los cuales aplicaron la SPE para recuperar proantocianidinas en heces de ratas a las que habían administrado un extracto de pepitas de uva. No obstante, y a pesar de esta optimización, en los siguientes experimentos tanto con soluciones de patrones puros como con soluciones fecales, encontramos que la eficacia de la SPE en la recuperación de algunos compuestos de interés como el ácido 4-*O*-metilgálico, el ácido siríngico y el ácido 4-hidroxi-5-(3',4'-dihidroxifenil)valérico era relativamente baja (<85%). Choy y col. (2013) obtuvieron conclusiones similares al aplicar la SPE a muestras fecales de ratas, encontrando restricciones en la recuperación de algunas proantocianidinas, principalmente aquellas con un grado de polimerización mayor. En consecuencia, estos resultados pusieron de manifiesto la no necesidad de utilizar la SPE como tratamiento previo de la muestra para el análisis de compuestos fenólicos por UPLC -ESI-TQ MS/MS en muestras fecales. El paso siguiente consistió en el estudio del perfil de metabolitos fenólicos en las muestras fecales humanas procedentes del estudio de intervención (Publicación 5.1.2). Aunque las principales técnicas utilizadas para el análisis de metabolitos fenólicos en fluidos biológicos humanos son la cromatografía de gases acoplada a la espectrometría de masas (Gonthier y col., 2003; Jenner y col., 2005; Muñoz-González y col., 2011) y la cromatografía de líquidos

(HPLC) acoplada a espectrómetrometría de masas (Medina-Remón y col., 2009; Vitaglione y col., 2013, de Ferrars y col., 2014), la separación y la sensibilidad de estas metodologías podría mejorarse con la utilización de cromatografía de líquidos de ultra alta eficacia (UPLC) para la separación, la cual permite trabajar con columnas de menor diámetro interno y partículas de relleno más pequeñas (<2µm), permitiendo trabajar a una mayor presión (más de 15 000 psi) y permitiendo, por tanto, una mayor rapidez en el análisis. La idoneidad de la metodología de UPLC-ESI-TQ MS/MS, utilizada en la publicación 5.1.2, para el análisis de metabolitos fenólicos derivados del metabolismo microbiano en muestras de heces generadas en estudios de intervención en humanos y de estudios de fermentación *in vitro* fue previamente demostrada (Sánchez-Patán y col., 2011).

El análisis de metabolitos fenólicos en las muestras fecales procedentes del estudio de intervención permitió cuantificar un total de 35 compuestos fenólicos diferentes, encontrándose, como cabía esperar, un mayor número de ellos en las muestras recogidas tras la intervención con vino respecto a las muestras basales, recogidas tras el periodo de lavado. Además, el contenido total de metabolitos fenólicos fue significativamente mayor tras de la ingesta de vino (625 ± 380 µg/g de heces, media±DS), en comparación con las muestras basales (358 ± 270 µg/g de heces). Es decir, el consumo moderado y regular de vino parecía promover el metabolismo de los compuestos fenólicos en el tracto digestivo. Un estudio piloto previo (n=8) de intervención en humanos con vino tinto (272 mL /día, 20 días) (Jiménez-Girón, 2013) también mostró un aumento significativo en el contenido total de metabolitos fenólicos tras la ingesta de vino, si bien sólo cuantificaron 22 metabolitos fenólicos tras la ingesta de vino, a pesar de que utilizó la misma metodología UPLC-ESI-TQ MS/MS.

Los valores de medios de concentración de metabolitos fenólicos en heces al inicio del estudio eran comparables a los rangos de concentración reportados por otros autores (Jenner y col., 2005; Karlsson y col., 2005; Gao y col., 2009; Gill y col., 2010; Gao y col., 2010; Knust y col., 2006), a pesar de las diferentes metodologías utilizadas, el diferente número de voluntarios, y la diferente metodología de preparación de la muestra. Al igual que estos autores, se encontró una alta variabilidad interindividual, lo que evidencia la dificultad que supone establecer valores de referencia en cuanto al contenido fecal de metabolito fenólicos. De ahí la necesidad de llevar a cabo estudios con un número alto de voluntarios y de establecer dietas estandarizadas y controladas, como se ha intentado hacer en este estudio.

Tras el periodo de ingesta de vino, se encontraron diferencias significativas (P<0,05) en el contenido fecal de 10 metabolitos fenólicos (ácido 3,5-dihidroxibenzoico, ácido protocatéquico, ácido 3-O-metilgálico, ácido vainíllico, ácido 3-hidroxifenilacético, ácido siríngico, ácido 4-hidroxi-5-(3',4'-dihidroxifenil)-valérico, y 4-hidroxi-5-fenilvalérico). Algunos autores, han reportado un aumento significativo en el contenido de algunos metabolitos fenólicos tras intervenciones dietéticas con otras fuentes ricas en polifenoles (Gill y col., 2010; Jacobs y col., 2008; Czank y col., 2013). Por ejemplo, Gill y col. (2010) reportaron los cambios en el contenido de metabolitos fenólicos tras una intervención nutricional en humanos (n=10) con un puré de frambuesa (200 g/día) durante 4 días, siendo el contenido de algunos de los metabolitos como ácido fenilacético, ácido 3-fenilpropiónico, ácido 3-hidroxifenilacético, y ácido 3-(4'-hidroxifenil)-propiónico equiparables a los obtenidos en la publicación 5.1.2.

La alta variabilidad individual encontrada en relación al contenido fecal de metabolitos fenólicos de origen microbiano en las heces podría indicar que la degradación de los precursores fenólicos estaría sujeta a la variabilidad inter-individual en cuanto a la composición y funciones de la microbiota intestinal. Por ejemplo, Sánchez-Patán y col. (2012) atribuyeron la diferente capacidad de degradación de los flavan-3-oles a las diferencias en la actividad esterasa de la microbiota intestinal. Los resultados de la publicación 5.1.2 mostraron diferencias especialmente notables entre voluntarios en el contenido fecal de los ácidos 4-hidroxi-5-(3',4'-dihidroxifenil)-valérico, 4-hidroxi-5-fenilvalérico y 5-(3'-hidroxifenil)-γ-valerolactona, metabolitos considerados como intermediarios del catabolismo de los flavan-3-oles y claves para explicar las diferencias individuales entre voluntarios así como el grado de catabolismo de los flavan-3-oles. De hecho, el compuesto 5-(3'-hidroxifenil)-γ-valerolactona ha sido previamente sugerido como un posible biomarcador de la ingesta de flavan-3-oles (Llorach y col., 2010). Sin embargo, apenas existe información acerca de las posibles bacterias responsables del catabolismo de los flavan-3-oles. Hasta el momento la capacidad de degradación de flavan-3-oles ha sido atribuida a algunas especies como *Eubacterium* sp. SDG-2, *Eggerthella lenta* y *Flavonifractor plautii*, que poseen actividades enzimáticas complementarias para la degradación de flavan-3-oles (Jin y Hattori, 2012; Kutschera y col., 2011; Wangetal y col., 2001). Otros autores, como Tabasco y col. (2001) y más recientemente Barroso y col. (2013), demostraron la capacidad de *L. plantarum* IFPL935 para degradar monómeros de flavan-3-oles vía la formación del intermediario difenilpropanol.

A pesar de la gran variabilidad interindividual encontrada, las diferencias en el contenido fecal total de metabolitos fenólicos tras la ingesta de vino permitieron clasificar a los voluntarios en tres grupos: metabolizadores altos, metabolizadores moderados y metabolizadores bajos. En este ámbito, otros autores también han propuesto clasificar a los sujetos como bajos, moderados y altos metabolizadores, teniendo en cuenta los niveles generados de algún metabolito fenólico específico en orina, tras intervenciones con fuentes ricas en precursores fenólicos (Truchado y col., 2012; Bolca y col., 2013).

La publicación 5.1.2 ha demostrado, por tanto, la diferente capacidad humana para metabolizar los polifenoles, en base al perfil fecal de metabolitos fenólicos tras la ingesta de vino. Sin embargo, este estudio ha puesto de manifiesto, una vez más, los problemas inherentes a la realización de estudios de intervención nutricional en humanos, a destacar, la enorme dificultad para estandarizar a la población de estudio y la consiguiente variabilidad en los resultados obtenidos. Esta variabilidad podría evitarse con la utilización de modelos *in vitro* adecuados, que permitieran tener un mayor control de las variables. De ahí surgió la necesidad de evaluar, *in vitro*, el efecto de la ingesta de vino sobre la microbiota humana (oral y colónica) mediante el uso de modelos de simulación capaces de simular, en gran medida, las condiciones fisiológicas de lo que ocurre en determinados segmentos del sistema digestivo, concretamente de la cavidad oral (publicación 5.3.1) y del tracto gastrointestinal (publicación 5.3.2), estudios que serán discutidos más adelante.

Los resultados encontrados sobre la capacidad del vino tinto de promover el metabolismo fenólico intestinal, aumentando significativamente el contenido de metabolitos fenólicos en la materia fecal, nos llevaron a plantear un estudio metabolómico de las heces con el objetivo de comprobar si también podía afectar al metabolismo de otros componentes (no fenólicos) y de otros nutrientes. Pese a que el interés de la metabolómica para el descubrimiento de nuevos biomarcadores de exposición dietética está cobrando gran interés (Scalbert et al., 2014), en el caso del vino se trata de un área muy poco explorada.

Con el objetivo de dilucidar el perfil metabolómico fecal, se desarrolló un método de análisis no dirigido mediante UHPLC-TOF MS, de las muestras fecales procedentes de los participantes en el estudio de intervención nutricional con vino. Los resultados confirmaron los descritos anteriormente mediante el análisis dirigido de metabolitos fenólicos (publicación 5.1.2.), y proporcionaron

conclusiones relevantes sobre los cambios en el metaboloma fecal, apartando nuevos datos acerca de otros metabolitos procedentes de otras vías metabólicas que podrían estar también relacionadas con la salud humana.

Aunque, como era de esperar, se encontró una enorme variabilidad intra- e inter-individual al analizar el metaboloma fecal de los voluntarios, fue posible detectar cambios significativos en 37 compuestos, de los cuales 23 mostraron una mayor respuesta tras la ingesta de vino mientras que 14 mostraron una respuesta menor. De los 37 metabolitos que mostraron diferencias tras la ingesta de vino, 20 pudieron ser tentativamente identificados. Algunos de estos compuestos identificados correspondieron a metabolitos exógenos, procedentes del vino, cuya presencia se confirmó bien mediante el análisis del vino utilizado en la intervención (2-ácido hidroxiglutárico) o bien porque ya habían sido reportados en la literatura (2-ácido hidroxiglutárico, 2-metilbutírico, 2,3-pentanodiona, malonato de dietilo, 2-fenetilo butirato, y hexanoato de 2-feniletilo). Entre ellos, el ácido 2-metilbutírico fue previamente reportado en heces (Gao y col., 2010). Como era de esperar, el contenido fecal de estos compuestos aumentó significativamente tras la ingesta de vino.

Otros de los compuestos identificados fueron los clasificados como metabolitos de origen microbiano (5 (3',4'-dihidroxifenil)-γ -valerolactona, 4-hidroxi-5-(3'-hidroxifenil)valérico, ácido benzoico y 4-hidroxi-5-(fenil)valérico), los cuales aumentaron tras ingesta de vino, poniendo de nuevo de manifiesto que el metabolismo fenólico es promovido por la ingesta de vino, al igual que se demostró en la publicación 5.1.2. Como se ha discutido previamente, las fenil-γ-valerolactonas y ácidos valéricos son metabolitos derivados exclusivamente del catabolismo microbiano de los flavan-3-oles (Monagas y col., 2010) y, como se esperaba, aumentaron con la ingesta de vino. Por el contrario, la ingesta de vino provocó una disminución en el contenido del 3-(3-hidroxifenil)propiónico, metabolito microbiano derivado también del catabolismo de flavan-3-oles, lo que concuerda también con lo encontrado en la publicación 5.1.2., pudiendo deberse dicha disminución a la actividad hidrolítica de la microbiota intestinal.

Por último, están los compuestos identificados y clasificados como metabolitos endógenos y/o compuestos derivados de rutas metabólicas de nutrientes no fenólicos. Entre ellos, se encontró una menor respuesta de xantina, base purínica que participa como metabolito intermediario en la

degradación del adenosin monofosfato en ácido úrico. Ésta se forma por la oxidación de la hipoxantina a peróxido de hidrógeno y radical superóxido por acción de la xantina oxidasa (XO). Su menor respuesta tras la ingesta de vino concuerda con estudios previos que sugieren que las propiedades antioxidantes de algunos compuestos bioactivos como los flavonoides (Da Silva y col., 2004) y el resveratrol (Acquaviva y col. 2002; Li y col., 2006) se deben a su efecto de inhibición sobre la actividad de la xantina oxidasa (XO). También identificó el ácido glutárico, reportado previamente en heces (Gao y col., 2010), cuyo respuesta fue mayor tras la ingesta de vino. Es un ácido dicarboxílico producido durante el metabolismo de algunos aminoácidos como lisina y triptófano. Además puede ser producido por las bacterias anaerobias del intestino (junto con el ácido láctico) (Wendel y col., 1995).

También fueron identificados el urobilinógeno y la estercobilina, subproductos de la degradación de la bilirrubina. El urobilinógeno se forma por la acción de algunas especies bacterianas intestinales, especialmente *Clostridium* spp. (Becker y col., 2011) a partir de la bilirrubina, conjugado biliar. Parte del urobilinógeno es reabsorbido y excretado por la orina. Sin embargo, el urobilinógeno restante en el intestino (estercobilinógeno) se oxida a estercobilina marrón, responsable del color característico de las heces. Estos metabolitos mostraron una menor respuesta tras la ingesta de vino, los que está en consonancia con varios estudios que defienden que los alimentos ricos en flavan-3-oles, como el vino, podrían modular la microbiota intestinal dando lugar a un incremento de las bacterias beneficiosas, e inhibiendo otras como *Clostridium* spp (Tzounis y col., 2011; Viveros y col., 2011; Martin y col., 2012; Cueva y col., 2013).

Además, el consumo de vino condujo a una mayor respuesta del ácido docosahexaenoico en las heces, ácido graso poliinsaturado omega-3 sintetizado de forma endógena y asociado con numerosos efectos potenciales para la salud. Algunos autores han sugerido que los polifenoles del vino podrían proteger a los ácidos grasos poliinsaturados omega-3, tales como DHA, de la oxidación en el plasma (Cazzola y Cestaro, 2011). Además las interacciones favorables entre flavan-3-oles y la microbiota intestinal podría promover el crecimiento de algunos grupos de bacterias intestinales beneficiosas del genero *Lactobacillus* sp. (Cueva y col., 2013), las cuales podrían tener un papel esencial en la absorción de los ácidos grasos poliinsaturados (Laparra y col., 2010).

Las enzimas de la microbiota intestinal están involucradas en el metabolismo de colesterol y sus metabolitos, los ácidos biliares. En el presente estudio se encontró que los ácidos biliares secundarios ácido desoxicólico y ácido sulfolitocólico, y el sulfato de colesterol fueron regulados por la ingesta de vino. Han y col. (2009) demostraron el efecto de supresión de algunos polifenoles (incluyendo la curcumina, ácido cafeico, catequina, rutina, y ácido elágico) sobre los niveles fecales de ácido desoxicólico y ácido litocólico en ratas a las que se había administrado una dieta rica en grasas, debido a la supresión de la biosíntesis de los ácidos biliares a partir del colesterol. Por otra parte, la transformación de los ácidos biliares primarios a ácidos biliares secundarios en el intestino es llevada a cabo, principalmente, por bacterias anaerobias, incluyendo los géneros *Clostridium* spp. (Nicholson y col., 2012), los cuales son inhibidos por fuentes ricas de flavan-3-ol (Tzounis y col., 2011; Viveros y col., 2011; Martin y col., 2012; Cueva y col., 2013). En línea con esto, se observó una disminución en el contenido de ácido sulfolitocólico en las heces tras la ingesta de vino. Por el contrario, se observó una mayor respuesta en el contenido de ácido desoxicólico, algo también observado por Montilla y col. (2004) cuando se suministró vino tinto como complemento a una dieta alta en colesterol en ratas hipercolesterolémicas. Estos autores observaron que la excreción de ácidos biliares fecales, así como sus concentraciones plasmáticas y hepáticas incrementaron significativamente, lo que podría indicar un aumento en la degradación del colesterol. Por último, Shimizu-Ibuka y col. (2009) estudiaron el efecto hipocolesterolémico de la piel de cacahuete administrada a ratas y revelaron que ésta tenía un fuerte efecto en la reducción del nivel de colesterol sérico y poco efecto sobre el nivel de colesterol hepático, aumentando la cantidad total de colesterol en las heces. Este estudio sugiere que los polifenoles del extracto habrían tenido un efecto anti-hipercolesterolémico por inhibición de la absorción del colesterol exógeno y lípidos en el intestino delgado. Los resultados de la publicación 5.1.3 también mostraron un aumento en la respuesta de sulfato de colesterol tras la ingesta de vino.

En conclusión, el método propuesto en la publicación 5.1.3 ha demostrado ser adecuado para evaluar los cambios en el metaboloma fecal tras la ingesta de vino. En nuestro conocimiento, este estudio ha sido el primero en la literatura que ha mostrado el perfil metabolómico en heces humanas derivado del consumo de vino tinto. Los resultados sugieren que algunos compuestos del vino llegan intactos a las heces mientras que otros son generados por la microbiota intestinal a partir de los

precursores fenólicos del vino. Además, la publicación 5.1.3 aporta información muy novedosa acerca del impacto del consumo de vino sobre otras rutas metabólicas de nutrientes no fenólicos.

En general, las diferencias observadas en el metaboloma fecal tras el consumo de vino podrían reflejar diferencias en la composición de la microbiota y su funcionalidad, que a su vez podrían atribuirse, en parte, a la modulación de esta por los polifenoles del vino y/o sus metabolitos. La realización de estudios metabolómicos y metagenómicos en paralelo permitiría estudiar, en conjunto, el metabolismo del huésped y de su microbiota con un gran detalle, ya que el metaboloma se ve afectado por el co-metabolismo y las interacciones metabólicas entre las bacterias del intestino y las células intestinales humanas (Chen y col., 2012).

Como ya se ha introducido previamente, para estudiar los efectos del vino y sus compuestos fenólicos sobre la salud digestiva es esencial tener en cuenta el papel que juega la microbiota intestinal, la cual cumple numerosas funciones beneficiosas para el huésped, marcadas por su espectro de actividades enzimáticas, participando activamente en el mantenimiento de la homeostasis y la función intestinal.

La composición de la microbiota intestinal es muy compleja y puede ser muy variable incluso entre sujetos sanos. Por tanto, cabe esperar que perfiles diferentes en cuanto a la composición de microbiota estén asociados a funciones enzimáticas diferentes y, por consiguiente, a una capacidad diferente en la metabolización de los nutrientes de la dieta. Por ello, y con el objetivo de comprobar si las diferencias entre voluntarios, en cuanto al metabolismo de polifenoles, encontradas en los estudios precedentes (publicación 5.1.2) pudieran deberse a una diferente composición de su microbiota intestinal, y de comprobar si, el consumo de vino tinto y su efecto sobre la salud humana podría estar relacionado no sólo con el aumento de metabolitos fenólicos sino también con cambios en el perfil específico de microbiota del sujeto que lo consumiera, nos planteamos el estudio de la composición de la microbiota intestinal en las heces de algunos voluntarios que habían participado previamente en el estudio de intervención dietética con vino, en concreto de aquellos voluntarios previamente clasificados según su diferente capacidad metabólica, como metabolizadores bajos, moderados o altos de polifenoles (Publicación 5.1.2) .

Por tanto, la publicación 5.2.1 (en preparación) tuvo como objetivo realizar un estudio de la composición de las poblaciones bacterianas intestinales mediante un análisis metagenómico de las heces de los voluntarios participantes en el estudio de intervención dietética con vino que mostraron diferente capacidad de metabolizar los polifenoles del vino (alta, moderada y baja). Esta publicación reveló que, tras el consumo de vino, no se encontraron diferencias significativas en la composición de las poblaciones microbianas, salvo en el caso de los géneros *Blautia* y *Proteus*, cuyos porcentajes relativos fueron significativamente menores en el grupo de metabolizadores bajos. Sin embargo, al comparar (test ANOVA y LSD) la composición bacteriana de las muestras fecales basales, antes de la intervención, entre los tres grupos de voluntarios (metabolizadores de polifenoles bajos, moderados y altos) se encontró que, a nivel de filo, en el grupo de metabolizadores altos la composición en *Actinobacterias* fue significativamente mayor, mientras que la de *Firmicutes*, sin ser significativa ($p=0.058$), fue apreciablemente menor. De forma relevante, el análisis de los datos a nivel de género mostró que, en el grupo de metabolizadores altos, hubo un porcentaje relativo significativamente mayor para el género *Bifidobacterium*. En un estudio piloto previo de intervención con vino (n=9), Boto-Ordoñez y col. (2014) comprobaron un mayor incremento en la población de *Bifidobacterium* en las heces de los sujetos que mostraron mayores cambios en el perfil de metabolitos fenólicos derivados de las antocianinas en orina (ácido siríngico, ácido *p*-cumárico, ácido 4-hidroxibenzoico y ácido homovainillínico) tras la ingesta de vino. Las especies que engloba el género *Bifidobacterium* son consideradas como especies beneficiosas que intervienen sobre la salud humana a diferentes niveles. Por un lado, han demostrado mejorar la función de la barrera intestinal, estimular el sistema inmune intestinal, además de prevenir procesos diarreicos o alergias y participar en la síntesis de provitaminas y el metabolismo de lípidos (Gibson y col., 2008 y Burcelin y col., 2012). Además, algunas especies del género *Bifidobacterium* tienen un importante papel de protección ya que son capaces de ejercer un efecto antimicrobiano frente a bacterias patógenas mediante la producción de ácidos grasos de cadena corta (AGCC) a la vez que compiten con ellas por los nutrientes y los sitios de adhesión (Gibson y col., 1994).

Por último, llevamos a cabo un análisis más avanzado a nivel de especie, lo que permitió encontrar diferencias significativas en la composición bacteriana basal, entre los tres grupos de voluntarios con diferente capacidad metabólica, en 19 especies bacterianas. Cabe destacar que dos especies

pertenecientes al género *Bifidobacterium* (*Bifidobacterium ruminantium* y *Bifidobacterium* sp. 113) mostraron un porcentaje relativo significativamente mayor en el grupo de los metabolizadores altos, lo que podría confirmar que dichas especies podrían estar relacionadas, al menos en parte, con el metabolismo de polifenoles.

Por otro lado, las especies *Uncultured Clostridales Family XIII bacterium* y *Clostridiales bacterium 10-3b*, mostraron ser significativamente menores en el grupo de los metabolizadores altos. Además, las especies *Clostridiales bacterium 10-3b*, *Staphylococcus aureus*, *Clostridium sp. enrichment culture clone VN_TX2-19* y *Uncultured Staphylococcus sp.,* fueron significativamente menores en los grupos de metabolizadores moderados y altos, respecto al grupo de metabolizadores bajos. Algunas especies del género *Clostridium* han sido asociadas con el desarrollo de enfermedades inflamatorias intestinales (Rastall y col., 2005). Es conocido que muchas cepas de *C. difficile* son productoras de toxinas causantes de diarreas severas (Ozaki y col., 2004), y cáncer de colorrectal mientras que algunas de actividades enzimáticas de *C. histolyticum* se han relacionado con el crecimiento de células neoplásicas y algunos tipos cáncer (Hambly et al., 1997).

Varios estudios *in vitro* e *in vivo* han indicado que los polifenoles del vino podrían modular selectivamente el crecimiento de algunas poblaciones bacterianas intestinales (Tzounis y col., 2008; Selma y col., 2009; Viveros col., 2011; Hidalgo y col., 2012; Queipo-Ortuño y col., 2012; Cueva y col., ; Parkar y col., 2013; Sánchez-Patán y col., 2013; Kemperman y col., 2013). En este sentido, cabe destacar que Queipo-Ortuño (2012), evaluó *in vivo* el efecto del vino sobre las poblaciones bacterianas intestinales, revelando un incremento en la población de Bifidobacterias tras el consumo de vino. En los últimos años se ha avanzado mucho en el reconocimiento del importante papel que juega la microbiota intestinal en el mantenimiento de la salud del organismo y en la prevención de patologías crónicas. Como ya hemos avanzado previamente, la microbiota intestinal participa en el mantenimiento de la homeostasis intestinal y, en condiciones fisiológicas normales, interacciona con las células intestinales del huésped y ayuda a mantener la integridad de la mucosa intestinal y del sistema inmunitario, protegiendo al huésped frente a la enfermedad.

Una microbiota alterada, por tanto, podría estar relacionada con el desarrollo de determinadas enfermedades relacionadas con el sistema inmune intestinal como enfermedades inflamatorias

intestinales (EII) y enfermedad celíaca, entre otras (Sanz y col., 2007; Salzman y Bevins, 2008). Aunque la etiología de las EIIs no está del todo clara, cada vez existen más evidencias que relacionan la microbiota intestinal con el desarrollo de estas enfermedades como consecuencia de un desequilibrio en las poblaciones bacterianas, favoreciendo el crecimiento de patógenos frente a bacterias con función protectora (Galvez y col., 2005; Rastall y col., 2005)

Dada la posible intervención de los polifenoles del vino en la modulación de la microbiota intestinal, podría sugerirse que los polifenoles del vino y sus metabolitos microbianos podrían tener un efecto indirecto en el mantenimiento de la homeostasis intestinal y en su función inmune. Además, algunos estudios *in vitro* han demostrado que los polifenoles podrían tener un efecto antinflamatorio, reduciendo la producción de algunos marcadores inmunes como citoquinas (Biasi y col., 2013; Bognar y col., 2013; García-Lafuente y col. 2009).

La publicación 5.2.2 surgió por tanto con el objetivo de evaluar el efecto del consumo moderado de vino sobre el estado de inflamación intestinal en individuos sanos. Para ello se analizaron los cambios en el perfil de marcadores inmunes, incluyendo inmunoglobulinas y citoquinas, con el fin de tener una visión lo más completa posible del estado inmunológico de los voluntarios participantes en el estudio de intervención en humanos, antes y después de la ingesta de vino. Como consecuencia del consumo de vino durante 4 semanas se encontró una disminución significativa en la concentración de 17 citoquinas en aquellos voluntarios que presentaban valores altos de citoquinas en heces al inicio de la intervención (n=6). Además esta disminución en el contenido de citoquinas fue especialmente destacable en aquellas citoquinas proinflamatorias o que promueven la inflamación inicial (TNF-α, IL-6, IL-8 e IFN-γ), las cuales se han relacionado con estados de inflamación y con el desarrollo de enfermedades inflamatorias del intestino (EII).

Estudios anteriores con modelos de inflamación con células colónicas han demostrado el efecto antiinflamatorio de algunos extractos de vino (Nunes y col., 2013; Angel-Morales y col., 2012; Romier-Crouzet y col., 2009). Nunes y col. (2013) estudiaron el efecto de un extracto de vino tinto en un modelo con células HT-29 estimuladas con citoquinas para promover el estado inflamatorio y demostró que el extracto de vino redujo significativamente la producción de IL-8. Por otro lado, Angel-Morales y col. (2012) utilizaron un extracto de vino tinto en un modelo de inflamación inducida en

fibroblastos de colon CCD- 18Co y observaron una disminución en la producción de IL- 6 y TNF-α. Otros autores han demostrado el efecto antinflamatorio (disminución en el contenido de citoquinas como IL-6 o IL-8) de algunos compuestos fenólicos del vino como malvidina (Bognar y col., 2013) o genisteína y (-)-epigalocatequina-3-galato (Sergent y col., 2010) en modelos *in vitro* con células humanas. Además Larrosa y col. (2009) y Sánchez-Fidalgo y col. (2010) encontraron importantes reducciones en la expresión de algunas citoquinas proinflamatorias (IL-1ß , IL-8 , IFN-γ y TNF-α) tras la administración de ácido hidrocafeico o resveratrol, respectivamente, en ratas tratados con DSS (inductor de un estado de colitis).

Dado el alto contenido fenólicos del vino utilizado y el aumento significativo de metabolitos fenólicos encontrados en la publicación 5.1.2, principalmente aquellos derivados del metabolismo de flavan-3-oles, cabría esperar que dichos metabolitos pudieran estar relacionados con el efecto antiinflamatorio encontrado en aquellos voluntarios con un nivel fecal inicial alto en citoquinas. Por tanto, la publicación 5.2.2 ha demostrado, por primera vez, que el consumo moderado de un vino tinto rico en polifenoles, especialmente en flavan-3-oles, podría modular favorablemente la respuesta inmune intestinal *in vivo* en huéspedes con inflamación intestinal asintomática.

Como se demostró en la publicación 5.2.1, el estudio metagenómico permitió discernir algunas diferencias en la composición de la microbiota en función del diferente perfil metabólico de los voluntarios. En este aspecto, cabe esperar, que dichos cambios pudieran tener también relación con el estatus de inflamación dado que la microbiota influye enormemente en este. Por ejemplo, el género *Bifidobacterium*, que resultó ser significativamente mayor en aquellos voluntarios con una capacidad alta de metabolizar polifenoles, tiene un importante papel en el mantenimiento de la integridad de la barrera intestinal y del sistema inmune, como se ha destacado previamente. Además, cabe destacar que los cambios en la microbiota intestinal producidos por el consumo de vino podrían además generar cambios en la producción de algunos productos de su metabolismo como los ácidos grasos de cardena corta o amonio. Los ácidos grasos de cadena corta, generados por la microbiota intestinal a partir de la digestión y el metabolismo de la fibra dietética podrían tener propiedades antiinflamatorias y prevenir, por tanto, el desarrollo de las EIIs, como algunos autores han sugerido (Galvez *y col*., 2005; Huda-Faujan *y col*., 2010; Vinolo *y col*., 2011). De hecho, algunos ensayos *in vitro* han revelado una disminución en la concentración de algunas citoquinas como IL-6, IL-8 y TNF-α en experimentos

realizados con células de colónicas de rata inflamadas, mediante tratamiento con DSS, e incubadas con algunos AGCC como acetato, propionato y butirato (Tedelind *et al*., 2007).

Por otro lado, como se puso de manifiesto en la publicación 5.1.2, el consumo de vino promovió el metabolismo fenólico y dio lugar a un aumento significativo de metabolitos fenólicos en las heces, especialmente aquellos procedentes del metabolismo de precursores del grupo flavan-3-oles como las valerolactonas o los ácidos valéricos. Por tanto, sería lógico pensar que el aumento en dichos metabolitos podría estar directamente relacionado con la disminución observada en el contenido fecal de citoquinas proinflamatorias en aquellos voluntarios con un perfil alto el citoquinas antes de la intervención.

6.2. Simulación oral del consumo moderado de vino tinto en un modelo de biopelícula de la placa supragingival humana: efecto sobre bacterias de la placa dental y metabolismo de polifenoles

A pesar de la enorme importancia y la necesidad de realizar estudios *in vivo*, que tengan en cuenta todas las variables y procesos que tienen lugar en el sistema digestivo, a la hora de evaluar el efecto de una intervención nutricional sobre dicho sistema, el empleo de modelos de simulación *in vitro* de los distintos segmentos que lo componen, podría ofrecer numerosas ventajas. Por un lado permitiría una evaluación inicial de los posibles efectos sin necesidad de utilizar sujetos vivos y las dificultades asociadas como la dificultad de cumplir las normas éticas así como la dificultad para obtener muestras. Por otro lado, el empleo de modelos de simulación reduciría de manera importante la variabilidad en los ensayos. Por ello, dentro de la presente tesis se han incluido dos publicaciones cuyo objetivo fue evaluar el efecto de la ingesta de vino y/o sus componentes, en modelos *in vitro* que simulan, por un lado, la placa supragingival dental y, por otro, el tracto gastrointestinal.

La publicación 5.3.1 tuvo como objetivo evaluar el efecto del vino tinto, vino desalcoholizado y algunos componentes de la matriz del vino como el etanol, los polifenoles, las manoproteínas y los péptidos sobre algunas bacterias orales asociadas en forma de biopelícula. Para ello se utilizó un modelo de biopelícula conformado por 5 especies comúnmente localizadas la placa supragingival (*A. oris*, *S. mutans*, *S. oralis*, *F. nucleatum*, *V. dispar*). *A. oris*, *S. oralis* y *V. dispar* fueron seleccionadas por ser colonizadores tempranos, que participan en las primeras etapas de formación de la biopelícula

dental. Estas especies son responsables de iniciar la formación de la placa dental bacteriana y permiten a los colonizadores secundarios la coagregación a la biopelícula (kolebrander y col. 2010). También se seleccionaron e incluyeron en el modelo dos colonizadores secundarios (*S. mutans* y *F. nucleatum*), el primero por su carácter cariogénico lo que le relaciona directamente con el desarrollo de la caries dental (Hamada y col., 1984) y el segundo por un papel esencial en la coagregación bacteriana dentro de la biopelícula gracias a su capacidad para generar adhesinas (kolebrander y col. 2010).

Los ensayos realizados con este modelo revelaron que las biopelículas tratadas con vino tinto y vino tinto desalcoholizado, sufrieron, visualmente (CSLM), una gran disminución en la viabilidad celular en comparación con aquellos tratados con agua (control negativo). Sin embargo, el tratamiento con la solución de extracto de vino no produjo cambios apreciables en la viabilidad celular. Además, los recuentos de unidades formadoras de colonias en placa revelaron reducciones significativas en las poblaciones de *F. nucleatum* y *S. oralis* tras el tratmiento con vino tinto y vino tinto desalcoholizado. Como se comprobó con el tratamiento con vino desalcoholizado, el etanol no fue el responsable de las propiedades antimicrobianas observadas. Además, para confirmar esto se evaluó el efecto del etanol tratando la biopelícula con una solución de 12% de etanol en agua. Este tratamiento provocó una disminución significativa en la población de *F. nucleatum*, pero no en la de *S. Oralis*. Estos resultados podrían indicar que el efecto del vino sobre a *F.nucleatum* podría deberse al etanol (aunque también se observó con vino desalcoholizado), mientras que el efecto sobre *S. oralis* podría deberse a otros componentes presentes en el vino, como los polifenoles.

El efecto del consumo de vino sobre la salud oral ha sido estudiado por varios autores, si bien con otras aproximaciones experimentales. Por ejemplo, Signoretto y col. (2010) llevaron a cabo un estudio epidemiológico con 75 voluntarios en el que analizaron mediante PCR-DGGE la placa supragingival y subgingival dental de voluntarios con distinto hábitos de consumo y concluyeron que *F. nucleatum* fue menos frecuente en los voluntarios que bebían de forma regular vino en comparación con los que bebían normalmente agua. Por otro lado, Daglia y col. (2009) demostraron las propiedades antimicrobianas de un vino desalcoholizado frente a algunos estreptococos orales. Nuestros resultados están de acuerdo con estos estudios en el hecho de que el vino inhibe selectivamente el crecimiento de algunas bacterias orales como *F. nucleatum* y *S. oralis.*

La publicación 5.3.1 también estudió la influencia de algunos componentes específicos del vino como los polifenoles, péptidos o manoproteínas de levadura. El tratamiento de las biopelículas con las soluciones de extracto de vino a las que se añadieron extractos ricos en algunos de los componentes inherentes al vino (extracto de semilla de uva, rico en flavan-3-oles, y extractos de levaduras secas inactivas, ricos en péptidos o manoproteínas) reveló que el tratamiento con la solución de extracto de pepita de uva provocó una reducción significativa en las poblaciones de *F. nucleatum*, *S. oralis* y *A.oris.* Zhao y col. (2014) demostraron también un efecto inhibidor y dosis-dependiente de un extracto de pepita de uva sobre formación de caries dental en un modelo de biopelícula mono-especie de *S. mutans*. Por otro lado, Cueva y col. (2012), reportaron también una inhibición significativa en el crecimiento de algunos estreptococos orales, tales como *S. mutans* y *S. sobrinus*, incubados con monómeros de flavan-3-oles como la (+)-catequina y la (-)-epicatequina. Igualmente Rotava y col. (2009) y Baydar y col. (2006) demostraron el efecto antimicrobiano de extractos de pepita de uva frente a bacterias patógenas tales como *S. aureus* y *E. coli* sugiriendo que la alta concentración de compuestos flavonoides y derivados en los extractos era la responsable de dicho efecto (Anastasiadi y col., 2009).

A pesar de que existen otros sistemas o modelos más novedosos, como los sistemas en cámara de flujo o los dispositivos intra-orales, para el estudiar las biopelículas orales y evaluar el efecto de tratamientos antimicrobianos sobre ellas, el modelo de biopelícula utilizado en la publicación 5.3.1 demostró ser adecuado para evaluar de forma eficaz la modulación de la microbiota oral asociada en forma de biopelícula por efecto del vino y sus componentes. Los resultados de la publicación 5.3.1 demostraron que el vino tinto inhibe el crecimiento de algunas especies patógenas en un modelo de biopelícula oral y que dicho efecto no es debido a su contenido en etanol. Además los prometedores resultados obtenidos con el extracto de pepita de uva, el cual mostró la mayor actividad antimicrobiana, permiten proponer dicho extracto como un ingrediente antimicrobiano potencial para la prevención de la caries dental.

6.3. Simulación gastrointestinal del consumo moderado de vino tinto en un nuevo modelo dinámico *in vitro* (SIMGI): avances en el metabolismo colónico de polifenoles

Por último, la publicación 5.3.2 tuvo como objetivo evaluar el efecto de la ingesta de vino sobre la microbiota intestinal y su metabolismo mediante el uso de un novedoso modelo *in vitro* de simulación gastrointestinal (SIMGI) el cuál demostró ser adecuado para tal fin.

Los modelos *in vitro* más utilizados para estudiar el efectos de una intervención nutricional sobre el sistema gastrointestinal son los sistemas de fermentación en *batch*, inoculados con cultivos puros de especies seleccionadas o con muestras fecales procedentes de voluntarios. Estos sistemas han sido muy utilizados en los últimos años para evaluar el efecto de los polifenoles del vino sobre la microbiota intestinal (Tzounis y col., 2008; Hidalgo y col., 2012; Cueva y col., 2012; Sánchez-Patán y col., 2012; Barroso y col., 2013). Sin embargo, dado que la actividad metabólica de la microbiota del colon es región-dependiente (Barroso et al., 2013), el abordaje de la interacción polifenoles-microbiota debe hacerse utilizando modelos de simulación que, de forma dinámica, monitoricen en el tiempo los cambios microbiológicos y metabólicos que tienen lugar en las diferentes partes del colon.

El modelo SIMGI (**SIM**ulator of the **G**astro**I**ntestinal tract) es un sistema desarrollado recientemente (Barroso et al., 2014). En este estudio, se ha utilizado este sistema para simular la ingesta aguda de vino tinto y evaluar su impacto en el metabolismo colónico.

Los resultados de la publicación 5.3.2 en relación al metabolismo fenólico revelaron que la ingesta de vino provocó un aumento en el contenido de algunos compuestos fenólicos como (+)-catequina, (-)-epicatequina y resveratrol en el colon ascendente y transverso, en primer lugar, y más tarde en el colon descendente, los cuales podrían proceder de la degradación de proantocianidinas (Monagas y col., 2010). De acuerdo a estos resultados, Van Dorsten y col. (2012) encontraron también un aumento inicial en el contenido de algunos de los polifenoles presentes inicialmente en un extracto de vino tinto utilizando un modelo SHIME. Como se había observado previamente en la publicación 5.1.2, tras la ingesta de vino hubo un incremento en el contenido de varios metabolitos intermedios, tales como difenipropan-2-ol y la 5-(3',4'-hidroxifenil)-γ-valerolactona, los cuales, como ya se ha discutido previamente, son considerados biomarcadores del metabolismo flavan-3-oles por parte la microbiota intestinal (Llorach y col., 2010; Sánchez-Patán y col., 2011). El mayor contenido de metabolitos

fenólicos se encontró, como se esperaba, en los compartimentos finales, es decir, colon transverso y descendente. En comparación con el vino sintético, se encontraron diferencias significativas para el ácido gálico, ácido protocatéquico, ácido 3-O-methylgallic, 4-hidroxibenzoico, ácido 3,4-dihidroxifenilpropiónico, ácido vanílico, ácido siríngico y ácido salicílico durante la ingesta de vino.

Respecto a la formación de amonio, los resultados mostraron que la ingesta de vino produjo una disminución gradual de este ion en todos los compartimentos, siendo más pronunciada en el colon ascendente, y llegando a ser estadísticamente significativa en el voluntario 1, lo que podría deberse tanto al agotamiento de las fuentes de nitrógeno, como consecuencia de la sustitución del medio nutritivo por vino (Chaikham y col., 2012), como a la capacidad de los polifenoles del vino para reducir la fermentación colónica de proteínas (Jacobs y col. 2012), lo que se asocia generalmente como un efecto beneficioso sobre la salud (de Wiele y col. 2004). Algunos estudios han revelado una disminución en la concentración de amonio (Kemperman y col. 2013; Barroso y col., 2014.) tras la administración de extractos de vino en un modelo SHIME.

Por otro lado, en cuanto al perfil de ácidos grasos de cadena corta (AGCC), los resultados mostraron que la ingesta de vino aumentó de forma notable la producción de ácido butanoico, especialmente en el colon transverso y descendente, lo que podría interpretarse como un efecto favorable para la salud ya que éste compuesto se ha asociado con efectos beneficiosos sobre la salud como la inhibición de la inflamación, disminución del estrés oxidativo y actividad anticancerígena (Scharlau y col., 2009; van Immerseel y col., 2010.). La publicación 5.3.2 reveló una tendencia al alza de la concentración de los AGCC, lo que concuerda con los resultados obtenidos en un estudio *in vitro* (Bialonska et al., 2010) en el que los autores observaron un aumento en la concentración de los ácidos acético, propiónico y butírico, en cultivos de microrganismos fecales humanos a los que se les adicionó un extracto de granada, siendo este aumento directamente relacionado con el potencial antinflamatorio de dicho extracto.

En cuanto al efecto de la ingesta de vino sobre la microbiota, los grupos bacterianos evaluados permanecieron relativamente estables durante y tras la ingesta de vino. Se apreció una disminución en el recuento de aerobios totales, resultado sin especial relevancia, ya que la mayoría de las bacterias presentes en el intestino son anaerobias, y de lactobacilos en el colon ascendente, así como un

aumento de estos últimos en el colon transverso, que podría explicarse con el aumento significativo de ácido butanoico en el colon transverso, ya que algunos lactobacilos, tales como *Lactobacillus acidophilus* y *Lactobacillus plantarum* IFPL935 son capaces de mejorar la producción de ácido butanoico (Chaikham y col., 2012;. Barroso y col., 2014.). Además cabe destacar que hubo una disminución en el colon ascendente del grupo *Bacteroides* y *All Bacteria*, siendo ésta última significativa, tendencia que fue también observada por Kemperman y col. (2013) y Barroso, y col. (2014) tras la administración de un extracto de vino tinto al SHIME.

En resumen, la publicación 5.3.2 demostró que la aplicación de vino tinto en el SIMGI generó cambios en la actividad metabólica de la microbiota colónica, determinados por la producción de diferentes metabolitos fenólicos, así como por una disminución en la producción de amonio y un aumento en la producción de ácido butanoico. A su vez, la actividad metabólica de la microbiota fue compartimento-dependiente e individuo-dependiente. Paralelamente, la ingesta de vino generó ligeros cambios a nivel de la composición de la microbiota, lo que concuerda con lo encontrado en la publicación 5.2.1.

Los resultados evidenciaron, de nuevo, una variabilidad en cuanto a la actividad metabólica de la microbiota procedente de los voluntarios donantes, teniendo una mayor capacidad metabólica el voluntario 1 respecto al voluntario 2, lo que está en conformidad con su clasificación anterior (Publicación 5.1.2) como metabolizador alto y metabolizador moderado, respectivamente.

En conjunto, los resultados de este estudio han demostrado la utilidad del SIMGI como modelo de simulación para evaluar los cambios metabólicos y microbianos que tienen lugar en las diferentes partes del intestino tras la intervención nutricional con un alimento.

CONCLUSIONES/CONCLUSIONS

7. CONCLUSIONES

Del presente trabajo, derivan las siguientes conclusiones:

1) El diseño planteado y la ejecución del estudio de intervención han permitido la comprensión integral de los efectos del consumo moderado de vino en el metabolismo de polifenoles y diversos aspectos relacionados con la función digestiva. Además, se han optimizado parámetros relativos al análisis de compuestos fenólicos en muestras fecales como la conveniencia o no de la extracción en fase sólida (SPE), calibración con patrón interno y dilución de la muestra.

2) El análisis por UPLC-ESI-TQ MS/MS ha permitido la identificación de hasta 35 metabolitos fenólicos en muestras fecales humanas procedentes del estudio de intervención, siendo la primera vez que algunos de ellos como los derivados de fenil-γ-valerolactona y de ácido 5-fenil-4-hidroxivalérico se describen en este tipo de muestras. Se ha observado una gran variabilidad inter-individual en el contenido de muchos de estos compuestos, atribuida a diferencias en la microbiota intestinal.

3) Además de metabolitos derivados de los polifenoles del vino, el análisis metabolómico por UHPLC-TOF MS de muestras fecales procedentes del estudio de intervención, identificó algunos compuestos del vino y metabolitos procedentes de otras rutas metabólicas, i. e. relacionados con el metabolismo de la bilirrubina, cuyo contenido variaba significativamente después del consumo moderado de vino durante 4 semanas. Estos resultados indican que la huella metabólica/metaboloma fecal asociada al consumo moderado y regular de vino, parece presentar compuestos que provienen del vino, así como otros biomarcadores de efecto asociados a rutas metabólicas que se ven afectadas por la ingesta del alimento.

4) El contenido total de metabolitos fenólicos en las muestras fecales después de la intervención con vino tinto, permitió clasificar a los voluntarios en tres grupos: metabolizadores altos (>1000 μg/g heces), metabolizadores moderados (500-1000 μg/g heces) y metabolizadores bajos (<500 μg/g heces), pudiéndose hablar de una estratificación de la población en función de su capacidad para metabolizar los polifenoles del vino, al igual que se ha descrito para otros alimentos.

5) El perfil microbiano (% relativo) obtenido del análisis metagenómico de un número seleccionado de muestras fecales correspondientes a los grupos de metabolizadores altos, moderados y bajos, no mostró diferencias estadísticamente significativas después de la ingesta para ninguno de los grupos, debido posiblemente al número relativamente bajo de muestras analizadas (5 muestras/grupo). Sin embargo, el perfil bacteriano de las muestras basales (antes de la intervención) resultó significativamente diferente en aquellos voluntarios que demostraron una mayor capacidad para metabolizar polifenoles, siendo mayor el porcentaje de Actinobacterias, a nivel de filo, de *Bifidobacterium*, a nivel de género, y de *Bifidobacterium ruminantium* y *Bifidobacterium sp. 113,* a nivel de especie. Por el contrario, los porcentajes de las especies*Clostridiales bacterium 10-3b*, *Staphylococcus aureus*, *Uncultured Staphylococcus sp*., y *Clostridium sp. enrichment culture clone VN_TX2-19*, consideradas como potencialmente patógenas, resultaron significativamente menores en los grupos de metabolizadores moderados y altos, lo que podría indicar que el aumento de metabolitos fenólicos en el lumen intestinal podría tener un efecto de modulación sobre la microbiota y, concretamente, sobre bacterias potencialmente patógenas.

6) Del total de 24 marcadores inmunes analizados, se encontró que el consumo de vino produjo una disminución estadísticamente significativa en el contenido de 16 citoquinas, la mayoría ellas consideradas como pro-inflamatorias, en aquellos voluntarios que mostraron contenidos de citoquinas altos al inicio del estudio. Se sugiere que el consumo moderado de vino tinto podría modular la respuesta inflamatoria intestinal *in vivo*, al menos determinada como el nivel de marcadores inmunes en heces.

7) Los tratamientos con vino tinto y vino tinto desalcoholizado resultaron eficaces frente a *F. nucleatum* y *S. oralis* en un modelo de biopelícula oral de la placa dental supragingival conteniendo 5 especies representativas de la microbiota oral. Hasta donde conocemos, este es el primer estudio sobre las propiedades antimicrobianas del vino en un modelo *in vitro* de biopelícula oral.

8) Se ha llevado a cabo la primera aplicación del sistema SIMGI a alimentos, en concreto, a vinos, comprobándose que la *capacidad relativa* de la microbiota fecal/intestinal para metabolizar los polifenoles del vino es similar entre los ensayos *in vitro* y el estudio de intervención, para los dos individuos seleccionados. Por otro lado, la generación de metabolitos fenólicos derivada de la alimentación del SIMGI con vino tinto, se relacionó con cambios significativos en la actividad metabólica y composición de la microbiota a nivel de grupos bacterianos.

7. CONCLUSIONS

The conclusions of this study are:

1) The design and implementation of the human intervention study have enabled a comprehensive understanding of the effects of moderate wine consumption in the metabolism of polyphenols and different aspects of the digestive function. In addition, parameters such the use or not of solid phase extraction (SPE), the use of internal standard calibration and sample dilution, have been optimized for the analysis of phenolic compounds in faecal samples.

2) The analysis by UPLC-ESI-TQ MS/MS allowed us the identification of up to 35 phenolic metabolites in the human fecal samples from the intervention study. Some of these compounds such as derivatives of phenyl-γ-valerolactone and of 5-phenyl-4-hydroxyvaleric acid were identified for the first time in faecal samples. A huge inter-individual variability in the content of many of these compounds was observed, and was attributed to differences in the intestinal microbiota.

3) Besides phenolic metabolites, the metabolomic analysis by UHPLC-TOF MS of the fecal samples from the intervention study allowed the identification of other compounds and metabolites from other metabolic pathways, for example those related to bilirubin metabolism, whose content varied significantly after the moderate consumption of wine during 4 weeks. The results showed that metabolic fingerprint or fecal metabolome associated with regular and moderate consumption of wine, appears to include wine compounds and other biomarkers associated with metabolic pathways that are affected by food intake.

4) The total content of phenolic metabolites in the fecal samples after the intervention with red wine, was used to classify volunteers into three groups: high metabolizers (> 1000 μg / g faeces), moderate metabolizers (500-1000 μg/g faeces) and low metabolizers (<500 μg/g faeces), we could be indicating that a stratification of the population according to their ability to metabolize wine polyphenols can be made, as has been described in the case of other foods.

5) The microbial profile (relative %) obtained from a metagenomic analysis of fecal samples from selected volunteers corresponding to groups of high, moderate and low metabolizers showed no statistically significant differences after intake for either groups, possibly due to the low number of samples analyzed (5 samples/group). However, the bacterial profile of baseline samples (before intervention) was significantly different in those volunteers who showed a greater ability to metabolize polyphenols (high metabolizers), with a higher proportion of Actinobacteria, at the edge level, *Bifidobacterium*, at the genus level, and *Bifidobacterium ruminantium* and *Bifidobacterium sp. 113* at the species level. On the contrary, the percentages of the species *Clostridiales 10-3b bacterium, Staphylococcus aureus, Staphylococcus Uncultured sp. And Clostridium sp. enrichment culture clone VN_TX2-19*, considered as potential pathogens, were significantly lower in the groups of moderate and high metabolizers, which may indicate that the ability to increase the luminal content of phenolic metabolites may have a modulating effect on the microbiota and specifically on potentially pathogenic bacteria.

6) Out of the 24 immune markers analyzed, it was found that wine consumption provoked a statistically significant decrease in the content of 16 cytokines, most of them considered as pro-inflammatory, in the volunteers who showed high contents of cytokines at baseline. It is suggested that the moderate consumption of red wine may modulate, at least at the level of immune markers in faeces, the intestinal inflammatory response *in vivo*.

7) The treatments with red wine and dealcholized red wine were effective against *S. oralis* and *F. nucleatum* in an oral biofilm model of the supragingival plaque containing 5 representative species of the oral microbiota. To our knowledge, this is the first study about the antimicrobial properties of wine in an *in vitro* oral biofilm model.

8) The application of the gastrointestinal simulator SIMGI to simulate food intake, in particular wine intake, has been carried out for the first time, confirming that the relative ability of faecal/intestinal microbiota to metabolize wine polyphenols was similar for both *in vitro* test and human intervention study, at least in the case of the two selected individuals. Moreover,

the production of phenolic metabolites derived from the wine intake simulation in the SIMGI was associated with significant changes in the metabolic activity and composition of the intestinal microbiota at the level of bacterial groups.

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8. BIBLIOGRAFÍA

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